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Functional characterization of the role of Bruno protein in translational regulation and germ line development in *Drosophila melanogaster*

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Dedication

To my parents,

who always support and encourage me

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Functional characterization of the role of Bruno protein in translational regulation and germ line development in *Drosophila melanogaster*

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Both body axes of the Drosophila egg are determined by localization of several mRNAs to specific regions within the oocyte. One of these mRNAs, *oskar (osk)*, is required for posterior body patterning. Localization and translational control are both crucial for the correct deployment of *osk*. Bruno (Bru) binds specifically to the 3'UTR of the *osk* mRNA and represses *osk* translation. In this dissertation, I first describe a genetic screen looking for dominant modifiers of the *arrest (aret)* mutant phenotype (*aret* encodes Bru). Two modifiers suggested additional targets for Bru action. One is *Star*, a gene that contributes to provision of Gurken activity. The second suggested target is a gene acting in the *Delta* signaling pathway. A final modifier, *Lk6*, encodes a protein kinase predicted to regulate eIF4E. I also took a biochemical approach trying to understand how Bru regulates *osk* translation. Bru protein contains three RNA Recognition Motifs, but the remainder of the protein had no known function. I identified

a domain, which is required for interaction to Bru itself, Cup and Apontic. Subsequent analysis of mutant forms of Bruno defective in these interactions led us to an unexpected discovery that Bru also acts as an activator of *osk* translation. Parallel analysis of Bru binding sites in *osk* 3'UTR fully support the notion that Bru has a dual role. There are two clusters of Bru Recognition Elements in either end of *osk* 3'UTR. Point mutations in one cluster cause overproduction of Osk protein while point mutations in the other cluster largely prevent translation of the message. To understand the molecular basis of the opposing roles of Bru, I used quantitative methods to better define and compare the binding of Bru to the different regulatory elements: those that either repress or activate *osk* mRNA translation. Using purified components I found that Bru binds to two clusters of binding sites in the *osk* 3'UTR differently, in terms of affinity, cooperativity and apparent compaction of the RNA. This work raises the possibility that the details of how Bru binds its substrate may determine whether it acts as a repressor or an activator.

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Chapter 1: General Introduction

This thesis is about post-transcriptional gene regulation during early development of *Drosophila melanogaster*. Both anteroposterior (AP) and dorsoventral (DV) body plans of the *Drosophila* egg are determined by localization of several mRNAs to specific regions within the oocyte (Davidson, 1986; Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999). One of these mRNAs, *oskar (osk)*, is required for posterior body patterning. *osk* mRNA is first synthesized very early in oogenesis, but Osk protein only accumulates later, after correct localization of the mRNA to the posterior pole of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1995; Kim-Ha et al., 1991). Ectopic activation of *osk* causes severe disruption of the anterior body plan (Ephrussi and Lehmann, 1992; Smith et al., 1992). Therefore, precise regulation of when and where Osk protein is made is crucial for correct patterning along the AP axis. A number of proteins have been shown to be important for localization or translational control of the *osk* mRNA. The first protein identified to play a role in *osk* translational control is Bruno (Bru). Bru binds specifically to the 3'UTR of the *osk* mRNA and represses *osk* translation (Castagnetti et al., 2000; Kim-Ha et al., 1995; Lie and Macdonald, 1999a; Webster et al., 1997). There are two major aims of research presented in this thesis. One is to determine the mechanisms by which Bru regulates *osk* translation, and the other is to identify additional mRNA targets of Bru and factors that may act upstream or downstream of Bru in the same pathway.

MATERNAL CONTRIBUTION IS ESSENTIAL FOR THE DEVELOPMENT OF THE EGG AND EARLY EMBRYO.

Maternal supply accumulates during oogenesis

Early animal development is controlled by maternally encoded RNAs and proteins, which are loaded into the egg during oogenesis (Davidson, 1986). When the eggs are laid, they can use those RNAs and proteins for general maintenance of the embryo to survive in the external environment until zygotic transcription begins, as well as for regulation of specific developmental processes in the embryo.

Oogenesis takes place in the female gonad, the ovary, and is the process of egg production. There are three cell types in the *Drosophila* ovary: oocyte, nurse cell and follicle cell. A typical *Drosophila* ovary contains 16-20 ovarioles. Each individual ovariole is composed of a string of progressively more mature egg chambers (King, 1970). In each egg chamber, one oocyte is connected to 15 nurse cells via cytoplasmic bridges called ring canals. Nurse cells synthesize large amount of RNAs and other cytoplasmic molecules that are transferred through the ring canals into the oocyte. Both oocytes and nurse cells are germ line cells, while follicle cells are somatic cells that surround the germ line cells. With the cytoplasmic contents provided largely by nurse cells during oogenesis, the oocyte becomes polarized while growing in size and eventually becomes an egg. Some of the contents of an egg are asymmetrically distributed before fertilization. This asymmetrical distribution is important for establishing the future body plan (Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999).

Maternally supplied factors are involved in body patterning

Some factors supplied by the mother have important regulatory roles in body patterning (van Eeden and St Johnston, 1999). These maternal factors are often

concentrated in particular regions of the egg. Following many rounds of cell divisions during development, they are inherited only by subsets of cells. Asymmetric distribution of these maternal factors allows them to either promote or suppress subsequent cellular events in certain regions, thus leading to the formation of future body pattern.

Many maternal-effect genes have been identified genetically that are required for specifying the body pattern (Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Schüpbach and Wieschaus, 1989). When these gene products are missing in the mother, the embryos produced display an abnormal body pattern. Some of these genes are acting in the same pathways, since loss-of-function mutants of these genes display similar defects (Schüpbach and Wieschaus, 1989). A small subset of these maternal patterning genes have special properties, and are called spatial determinants. The determinants are localized to specific regions of the egg or embryo, are required for patterning of that region, and can reprogram patterning if present at other regions (Driever et al., 1990; Ephrussi and Lehmann, 1992; Frohnhöfer et al., 1986; Gavis and Lehmann, 1992; Webster et al., 1994).

The *osk* gene encodes a posterior body patterning determinant (Lehmann and Nüsslein-Volhard, 1986). If the mother is homozygous mutant for *osk*, the embryos produced are defective in posterior patterning and have no abdominal segments. There are many other mutants that can give a similar phenotype. What sets *osk* apart from other genes involved in posterior body patterning is that *osk* has the ability to direct this patterning process. For example, when the mothers either have too much *osk* or have mislocalized *osk*, this ectopic *osk* activity reprograms anterior-posterior patterning and causes the loss of anterior structures. In strong cases, a mirror image of the posterior can form at the anterior and result in a symmetrical cuticle phenotype called bicaudal

(Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Smith et al., 1992). Because of this strong patterning activity of *osk*, careful regulation has to be in place to restrict *osk* activity to the posterior.

LOCALIZATION AND TRANSLATIONAL CONTROL OF THE *OSK* MRNA RESTRICT OSK ACTIVITY TO THE POSTERIOR

mRNA localization is a process that is prominently used in fly ovaries (St Johnston, 1995). Localization of mRNAs allows specific proteins to be synthesized in the subcellular regions where their activities are required and avoids regions where their expression is not desirable.

osk localization

osk mRNA appears very early in oogenesis and is concentrated in the oocyte cytoplasm. It becomes restricted to the posterior pole of the oocyte at stage 8/9, and remains there until early embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). The localization process involves the movement of the transcripts first into the oocyte from adjacent interconnecting nurse cells where the transcripts are made, and then across the length of the oocyte to the posterior pole. There is a transient appearance of the *osk* mRNA at the anterior boundary of the oocyte at stage 8, indicating that anterior accumulation is an intermediate step of the *osk* localization process.

Localized mRNAs often contain cis-elements in the 3'UTR that are required for localization (Johnstone and Lasko, 2001). In *Drosophila*, a number of maternal mRNAs have well characterized localization signals. Taking *bicoid* as an example, it is required for anterior body patterning and its mRNA localizes specifically to the anterior of the oocyte (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988; Frohnhöfer and Nüsslein-Volhard, 1986). The 3'UTR of *bicoid* mRNA contains 5 stem-loops (I-V), of which IV/V is sufficient for anterior localization in the ovary (Macdonald, 1990;

Macdonald and Kerr, 1997). The *osk* localization signal is more complex, in part because there is no strongly predicted secondary structure of the 3'UTR. Several elements were identified in the *osk* 3'UTR that are required for each of 3 different steps of localization: early transport into the oocyte, transient anterior accumulation and posterior localization (Kim-Ha et al., 1993). However, these elements have not been narrowly defined, and their properties remains largely uncharacterized.

The polarized microtubule network, which extends from nurse cells to the oocyte, mediates the bulk of *osk* mRNA transport during early stages of oogenesis (Brendza et al., 2000; Clark et al., 1994; Pokrywka and Stephenson, 1995; Theurkauf et al., 1992). Staufen is a protein that is made in nurse cells and colocalizes with the *osk* mRNA to the posterior pole of the oocyte where *osk* mRNA is anchored with the help of Osk protein, Staufen and BicD (Ephrussi et al., 1991; Kim-Ha et al., 1991; Micklem et al., 2000; Rongo et al., 1995; St Johnston et al., 1991; Swan and Suter, 1996; Vanzo and Ephrussi, 2002). Translation of the *osk* mRNA starts after the mRNA is localized at the posterior (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). The *osk* mRNA encodes two overlapping proteins, Long Osk and Short Osk, using alternative in-frame start codons (Markussen et al., 1995; Rongo et al., 1995). Long Osk anchors both mRNA and Short Osk at the posterior cortex of the oocyte (Vanzo and Ephrussi, 2002). Short Osk is required for the activity of *osk* in two important developmental processes, each at a different dosage level (Breitwieser et al., 1996; Markussen et al., 1995). A low level of Osk is required for directing posterior body patterning and a high level of Osk is required for pole plasm formation (Markussen et al., 1995; Rongo et al., 1995). Both of these processes depend on correct localization of *osk* mRNA: specific localization restricts *osk* activity to the posterior where it is required for posterior body patterning; localization

also provides positional information for pole plasm formation, which specifies the future germline (Ephrussi et al., 1991; Kim-Ha et al., 1991; Vanzo and Ephrussi, 2002).

The *osk* mRNA is concentrated in the oocyte throughout oogenesis while Osk protein is clearly absent at early stages (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). It is only when the *osk* mRNA is localized to the posterior pole of the oocyte at stage 8 that the Osk protein is detectable. By comparing the distribution of the *osk* mRNA and the Osk protein throughout oogenesis, one can easily tell that localization of the *osk* mRNA alone is not sufficient to restrict Osk activity to the posterior. Unlocalized *osk* mRNA has to be translationally repressed or the nascent Osk protein has to be quickly degraded to prevent ectopic accumulation of Osk protein. Furthermore, this negative regulatory mechanism must be limited temporally. It has to be in place from very early in oogenesis and be removed or overcome after localization of the mRNA is completed to then allow the protein to accumulate at the posterior. This tight coupling of localization and translational control is crucial for the deployment of *osk* (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995; Smith et al., 1992).

osk translational regulation

Two approaches were taken by various researchers to identify genes that play a role in translational regulation of *osk*. Genetically, one can look for mutants that will allow Osk to appear precociously. Alternatively, one can rely on biochemical analysis to see which protein(s) bind to the *osk* mRNA, and then determine if such a protein is required for localization or translational control.

The biochemical approach succeeded first and led to the discovery of a protein called Bru (Kim-Ha et al., 1995). Bru was identified in an UV crosslinking assay as an ovarian RNA binding protein that binds to the *osk* 3'UTR. There are two discrete regions

in the *osk* 3'UTR that Bru recognizes specifically: the AB region is located shortly after the coding sequence and the C region close to the poly(A) tail. Both of these regions contain multiple repeats of Bru Response Elements (BREs), U(G/A)U(A/G)U(G/A)U. Point mutations in the BREs abolish Bru binding.

Genetic analysis has also led to the identification of several other genes that are also involved in translational regulation of *osk* (Chang et al., 1999; Cook et al., 2004; Lie and Macdonald, 1999b; Micklem et al., 2000; Nakamura et al., 2001; Nakamura et al., 2004; Saffman et al., 1998; Wilson et al., 1996; Yano et al., 2004). Regulation can be negative or positive. I will introduce factors that are involved in negative regulation of *osk* translation first, including Bru and several other gene products. Then I will introduce another protein, Orb, which is involved in positive regulation of *osk* translation (Castagnetti and Ephrussi, 2003; Chang et al., 1999).

Bru and other gene products repress* osk *translation

To investigate what role Bru may play in the deployment of *osk*, a genomic *osk* transgene was made with mutations in all BREs in the 3'UTR to prevent Bru from binding and look for *osk* expression in the absence of Bru regulation (Kim-Ha et al., 1995). *osk* with such a mutant 3'UTR is transcribed and localized properly but the Osk protein accumulates abnormally: the Osk protein appears precociously throughout the oocyte cytoplasm during stage 7/8 and returns to normal (restricted at the posterior pole) as oogenesis proceeds, indicating that the mutant mRNA is partially translationally unregulated. Thus the Bru binding sites in the *osk* 3'UTR are crucial for translational repression of *osk*. A more direct form of evidence suggesting that Bru is a translational repressor came from studies carried out using an in vitro translation system with Drosophila embryo extract (contains no Bru) and purified Bru protein. A luciferase reporter RNA bearing a wild type *osk* 3'UTR is translationally repressed by Bru, while

the same reporter RNA bearing BRE mutant versions of the *osk* 3'UTR is unregulated (Castagnetti et al., 2000; Lie and Macdonald, 1999a). Both in vivo and in vitro evidence clearly demonstrate that Bru is required for translational repression of *osk*, and it does so by binding to the BREs in the 3'UTR.

In order to see if loss of Bru function will unleash *osk* translation in early stage oocytes, females carrying mutations in the gene that encodes Bru were analyzed. Cloning of the gene (Webster et al., 1997) revealed that it corresponds to the *arrest (aret)* gene, for which multiple mutations had been isolated (Schüpbach and Wieschaus, 1991). *aret* mutants have pleiotropic phenotypes, ranging from an early arrest of oogenesis to irregular embryonic segmentation defects (Schüpbach and Wieschaus, 1991; Webster et al., 1997). Neither intermediate nor weak *aret* mutants produce precocious Osk protein during oogenesis or show any body patterning defect in progeny embryos that could be associated with *osk* overexpression (Ephrussi and Lehmann, 1992; Smith et al., 1992). Strong *aret* mutants arrest oogenesis at a stage when the oocyte has not yet been specified, preventing detection of *osk* expression. Since *aret* mutants do not show an obvious defect in *osk* expression, and with extensive evidence supporting the conclusion that Bru represses *osk* translation, one needs to use other means to investigate how Bru function.

One approach is to study other proteins that Bru interacts with. Apontic (Apt) interacts with Bru in the yeast two-hybrid system and in vivo (Lie and Macdonald, 1999b). Embryos produced by females trans-heterozygous for *aret* and *apt* mutations exhibit anterior patterning defects. This phenotype can be suppressed by reduction of *nanos*, suggesting that it is caused by ectopic Osk. This genetic evidence suggests that Apt also has a role in regulating *osk* translation. Although Apt binds to the AB and C

regions of the *osk* mRNA 3' UTR, as does Bru, the binding of Apt is not as specific as it also binds to other sequences.

Another protein that interacts with Bru is Cup. The discovery of Cup is quite exciting because it leads to a model for Bru-dependent repression of *osk* translation (Nakamura et al., 2004; Wilhelm et al., 2003). The model suggests Bru-mediated repression happens at the level of initiation. Initiation is the rate-limiting step of translation, and therefore is often the target of many regulatory mechanisms (Gingras et al., 1999; Gray and Wickens, 1998). In cap-dependent translational initiation, the 5' cap structure of the mRNA attracts the eukaryotic initiation factor complex, eIF4F, to the mRNA. This complex contains the cap-binding protein eIF4E and the scaffold-like protein eIF4G. eIF4E binds to the 5' cap of the mRNA while eIF4G recruits the 40S ribosome subunit through eIF3. Translation initiation occurs when the eIF3-40S complex is recruited to the 5' end of the mRNA through an eIF4E-eIF4G-eIF3 interaction. The link between eIF4E and eIF4G is a key to translation initiation, and is a crucial target for repression. Initiation is disrupted by many eIF4E binding proteins (4E-BPs), which complete with eIF4G for binding to eIF4E and disrupt the eIF4G-eIF4E link (Gingras et al., 1999). The most relevant example is Maskin, which can be recruited to the 3'UTR of specific mRNAs and compete with eIF4G for binding to eIF4E (Mendez and Richter, 2001). Cup also contains a conventional eIF4E-binding domain and it can also act as an 4E-BP. In addition, Cup directly interacts with Bru (Nakamura et al., 2004). The model is that Cup can be recruited to the *osk* 3'UTR by Bru, where it binds to eIF4E and prevents it from binding to eIF4G. Due to local depletion of eIF4E, the initiation of *osk* translation is disrupted. Consistent with this model, *cup* mutant females display premature Osk expression at stage 6/7 and abnormal Osk distribution at stage 8/9. Cup may have

additional functions besides regulating *osk*, as strong *cup* mutants show defects in early oogenesis, which cannot be due to ectopic Osk.

Multiple repressive mechanisms are arranged both spatially and temporally to achieve precise regulation of *osk* translation. An example of spatial arrangement includes a protein called Me31B, which associates with the same RNP particles containing Cup, Bru, eIF4E and many maternal RNAs including *osk* (Nakamura et al., 2001; Wilhelm et al., 2000). *me31B* mutants display precocious Osk accumulation mostly in early stage nurse cells, as opposed to the oocyte accumulation of Osk observed in *cup* mutants, indicating that Me31B represses *osk* translation primarily in nurse cells. Bru/Cup mediated repression may be primarily required in the oocyte. This sequential arrangement demonstrates that multiple mechanisms are involved in repressing *osk* translation, and they can be arranged spatially to prevent ectopic Osk accumulation. For example, different parts of the *osk* RNP complex can repress *osk* expression at different segments of the transit. Alternatively, the *osk* RNP complex can change its composition along the transit, with some core component always associating with *osk* mRNA and other auxiliary factors present only when they are needed.

Not only is *osk* translation regulated spatially, it is also regulated temporally during oogenesis. Osk expression is repressed at all early stages prior to stage 8/9, but disruption of Bru/Cup mediated repression only depresses expression during stages 6-8 (Kim-Ha et al., 1995; Nakamura et al., 2004), which leaves the possibility that an alternative mechanism is required for repressing *osk* during at earlier stages. Indeed, other factors have been shown to regulate *osk* silencing during stage 1-6 (Cook et al., 2004). These factors that act early on *osk* are components involved in the RNAi pathway (Aravin et al., 2001; Findley et al., 2003; Kennerdell et al., 2002). *armi*, *spn-E*, *mael* and *aub* mutants display precocious Osk accumulation in very early stage (3/4) oocytes,

without dramatically affecting the level of *osk* mRNA. These mutants are also defective in polarization of the microtubule cytoskeleton and posterior localization of *osk* mRNA. Although proteins encoded by these 4 genes are components of the RNAi pathway, there is no evidence that they act on *osk* through the RNAi pathway. Nevertheless, it reveals a possibility that small RNAs can act as key regulators of *osk* translation during early oogenesis prior to Bru/Cup mediated repression. Consistent with this, *osk* mRNA in the ovary has been found to be associated with polyribosomes, even in the absence of localization of the mRNA or accumulation of Osk protein (Braat et al., 2004). Polyribosome association is the hallmark of mRNAs under miRNA control (Olsen and Ambros, 1999) and is another indication that small RNAs may be involved in *osk* translational control.

The last protein involved in *osk* repression that I need to introduce is Hrp48. Hrp48 is a protein that binds to the same regions in the *osk* mRNA 3'UTR as Bru does, but does not appear to directly interact with Bru. Hrp48 was identified as an abundant Drosophila heterogeneous nuclear RNA-associated protein. It contains two N terminal RNA binding domains (RBDs) and a C terminal Glycine rich domain (Matunis et al., 1992a; Matunis et al., 1992b). Several missense alleles of *hrp48* display a defect in *osk* RNA localization (without affecting the microtubule cytoskeleton) where the RNA is dispersed in the oocyte rather than localized to the posterior pole (Huynh et al., 2004). A transposon insertion mutant that reduces Hrp48 levels, but does not affect the structure of the protein, causes derepression of a reporter mRNA with all *osk* regulatory sequences. These is also genetic evidence suggesting that reduction of Hrp48 activity affects *osk* mRNA translation, but this may or may not involve binding of Hrp48 to the *osk* mRNA (Yano et al., 2004). Biochemical analysis showed that Hrp48 binds to the AB region in the *osk* 3'UTR in a UV crosslinking assay (Gunkel et al., 1998). The binding site for

Hrp48 is not well defined, although a shorter AB region containing only the central portion of AB (removes one BRE) reduces Hrp48 binding (Gunkel et al., 1998).

Orb is required for* osk *translation

Translational repression of *osk* has to be overcome after localization of the mRNA to allow the Osk protein to accumulate at the posterior. Little is known about how this is achieved except that two phases are likely to be involved in the transition: derepression and translational enhancement. One protein that is required for *osk* translational enhancement in the oocyte is Orb (Castagnetti and Ephrussi, 2003; Chang et al., 1999; Christerson and McKearin, 1994; Lantz et al., 1994). Orb is the Drosophila cytoplasmic polyadenylation element binding (CPEB) homolog (Lantz et al., 1992). CPEB proteins bind to the cytoplasmic polyadenylation element (CPE) of translationally repressed mRNAs and facilitate their polyadenylation and expression (Mendez and Richter, 2001). There is an U-rich element which resembles an CPE in the *osk* 3'UTR, but direct evidence of Orb binding to the U-rich element is still lacking.

Poly(A) tail elongation of a silenced mRNA can trigger translational initiation and maintaining a long poly(A) tail can be beneficial for persistent expression (Mendez and Richter, 2001; Paris and Richter, 1990). Three mRNAs that are involved in anterior, dorsal-ventral and terminal specification, *bicoid*, *toll* and *torso*, respectively, increase their poly(A) tail length concomitant with translation (Sallés et al., 1994). *osk* mRNA in wild type ovaries already has a long poly(A) tail, suggesting that the bulk part of the message is constitutively polyadenylated. Therefore, maintaining the poly(A) tail of *osk* mRNA at the posterior may be critical for Osk accumulation. In both strong and weak *orb* mutants, the *osk* mRNA poly(A) tail appears to be shorter, suggesting Orb is required for polyadenylation of the *osk* mRNA (Chang et al., 1999). Strong *orb* mutants arrest oogenesis early, preventing examination of the Osk protein level. One weak *orb* mutant,

orb^{MEL}, allows oogenesis to complete, but little or no Osk protein is detected at the posterior of the oocyte, indicating Orb is also required for *osk* translation (Chang et al., 1999). Therefore, Orb appears to act as a positive regulator of *osk* translation, and it may do so by polyadenylation of the *osk* mRNA.

It is important to point out that positive regulation by Orb is unlikely to be the decisive event in translational activation of *osk* at the posterior pole. It has been shown that a long poly(A) tail is not sufficient to overcome Bru-mediated repression in *osk* 3'UTR (Castagnetti and Ephrussi, 2003). Therefore polyadenylation cannot be the sole mechanism for derepression of *osk* translation at the posterior of the oocyte. Instead, polyadenylation is more likely to be required for enhancing *osk* translation after the translation is initiated (by a yet unknown mechanism); and allowing accumulation of Osk protein to levels sufficient to promote posterior patterning and germline formation (Castagnetti and Ephrussi, 2003). Furthermore, Orb and Bru have been shown to interact with each other in an immunoprecipitation experiment (Castagnetti and Ephrussi, 2003). It is not clear if the Bru:Orb interaction is RNA dependent, but the fact Bru and Orb interact suggests that both of them can be in the same RNP complex with *osk* mRNA (Chang et al., 1999). In addition, Bru binds to a cluster of BREs (the C region) in the *osk* 3'UTR that is close to the polyadenylation site (Kim-Ha et al., 1995). Although the significance of Bru:Orb interaction is unknown, one possibility is that Bru recruits Orb to the *osk* 3'UTR, then Orb polyadenylates *osk* mRNA. Since Bru and Orb act on opposite directions on *osk* translation, further studies on Bru:Orb interaction may help us understand how the negative and positive regulatory mechanisms are coordinated on the *osk* mRNA.

INSIGHTS FROM BRU HOMOLOGS

It is sometimes possible to gain insights into the function of a protein by looking at homologous proteins from other species, especially those that are well studied and have a clear function. Bru protein contains three RNA Recognition Motifs (RRMs). RRM1 and 2 are next to each other in the center of the protein while RRM3 is at the C-terminus. Many proteins have a similar organization of RRMs (Ladd and Cooper, 2004; Paillard et al., 1998; Timchenko et al., 1996). The RRMs are highly conserved, but the rest of the residuals in Bru homologs are largely divergent. Two proteins are particularly interesting: one provides insight on the target sequence Bru may bind to; and the other provides a clue on which part of the Bru protein may mediate its function.

EDEN-BP (embryo deadenylation element (EDEN)-binding protein) binds specifically to the EDEN motif in the 3'UTR of maternal mRNAs and targets these mRNAs for deadenylation and translational repression in Xenopus embryos (Paillard et al., 1998). EDEN-BP contains three RRMs and is closely related to Bru (Paillard et al., 1998; Webster et al., 1997). EDEN-BP binds to sequences containing multiple U(G/A) repeats or UGUA/UAUG tetra-nucleotides (Audic et al., 1998). Interestingly, a BRE, U(G/A)U(A/G)U(G/A)U (Kim-Ha et al., 1995), can be separated into two tetra-nucleotide elements. Given that an RRM contacts 2-4 nucleotides (Maris et al., 2005), it is conceivable that Bru can bind to two tetra-nucleotide elements in one BRE. This provides us with a new opportunity to re-examine Bru binding sites in the *osk* mRNA 3'UTR when we study how Bru function.

Another protein, Embryonic lethal abnormal vision (ELAV) type RNA binding protein 3 (ETR-3), provides an interesting clue about the function of the divergent region between RRM1/2 and RRM3. ETR-3 is a protein that also resembles the Bru protein domain structure. The region between RRM1/2 and RRM3 has recently been shown to be

important as the sequence within this region is involved in mediating the cytoplasmic localization of ETR-3 and splicing activity of the protein (Ladd and Cooper, 2004). This finding from ETR-3 presents a possibility that the same region in other proteins with similar domain structure are also required for their activities even though sequences in this region are not conserved. More details emerged from a subsequent study that systematically deleted short segments of this divergent region. They found that distinct residues within the divergent region are required for both activation and repression of different exons, and residues required for the repressive activity completely overlap with those required for activation (Han and Cooper, 2005). Although splicing and translational control are two very different processes, the function of the divergent region in ETR-3 certainly provides a clue for us to start looking at Bru function, especially when we know that the RRMs are required for Bru binding to the *osk* mRNA, but the function of other regions including the divergent region are completely unknown.

OVERVIEW OF THE DISSERTATION RESEARCH

There are two major aims of the research in this thesis, to identify additional targets of Bru and factors that may act upstream/downstream of Bru in the same pathway, and to understand the molecular mechanism of how Bru regulates *osk* translation.

To identify additional targets of Bru and/or other genes that act in the same pathway, I performed a genetic screen looking for dominant modifiers of the *aret* mutant phenotype (*aret* is the gene that encodes Bru) (Schüpbach and Wieschaus, 1991; Webster et al., 1997). Two of the modifiers suggested additional targets for Bru action. One modifier is *Star*, a gene that contributes to provision of Gurken activity (Gurken specifies the dorsal-ventral axis) (Ghiglione et al., 2002). Females heterozygous for $Star^{-}$ and homozygous for $aret^{-}$ produce excess Gurken protein, supporting the proposal by the Ephrussi lab that *gurken* is an additional target of Bru (Filardo and Ephrussi, 2003). The

second suggested target is a gene acting in the Delta signaling pathway. Females heterozygous for *Delta*$^{-}$ and homozygous for *aret*$^{-}$ display a phenotype very similar to that of homozygous *Delta* mutants (Lopez-Schier and St Johnston, 2001), and *aret* mutants have significantly reduced level of Delta protein at the interface of germline and follicle cells. A final modifier, *Lk6*, may prove to be informative about the mechanism of Bru-dependent translational repression. *Lk6* encodes a protein kinase predicted to regulate the rate-limiting initiation factor eIF4E (Lachance et al., 2002).

I also took a more biochemical approach in a quest of the mechanism by which Bru regulates *osk* translation. Bru protein contains three RRMs, but the remainder of the protein had no known function. I identified a domain (which we now call the interaction domain), that is required for interaction to three other protein tested – Bru itself (a novel finding of this study), Cup (Nakamura et al., 2004) and Apontic (Lie and Macdonald, 1999b). Subsequent analysis, including *in vivo* experiments, of mutant forms of Bru defective in these interactions led us to an unexpected discovery of a novel role of Bru protein – it also acts as an activator of *osk* translation. Parallel analysis of Bru binding sites in the *osk* mRNA 3'UTR fully supports the notion that Bru has a dual role. There are two clusters of BREs in *osk* mRNA 3'UTR (Kim-Ha et al., 1995). Point mutations in one cluster (close to 5' end of the 3'UTR) cause overproduction of Osk protein while point mutations in the other cluster (close to the polyadenylation site) largely prevent translation of the message.

In order to understand the molecular basis of the opposing roles of Bru, I used quantitative methods to better define and compare the binding of Bru to the different regulatory elements: those that either repress or activate *osk* mRNA translation. Using purified components I found that Bru binds to two clusters of binding sites in the *osk* 3'UTR differently, in terms of affinity, cooperativity and apparent compaction of the

RNA. This work raises the possibility that the details of how Bru binds its substrate may determine whether it acts as a repressor or an activator.

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Chapter 2: Genetic interactions of *Drosophila melanogaster arrest* reveal roles for translational repressor Bruno in accumulation of Gurken and activity of Delta

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Running head: Bruno regulates *grk* and *Dl*

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ABSTRACT

arrest mutants have pleiotropic phenotypes, ranging from an early arrest of oogenesis to irregular embryonic segmentation defects. One function of *arrest* is in translational repression of *oskar* mRNA; this biochemical activity is presumed to be involved in other functions of *aret*. To identify genes that could provide insight into how *arrest* contributes to translational repression, or that may be targets for *arrest*-dependent translational control, we screened deficiency mutants for dominant modification of the *arrest* phenotype. Only four of the many deficiencies tested, which cover ~30% of the genome, modified the starting phenotype. One enhancer, identified fortuitously, is the *Star* gene. *Star* interaction with *arrest* results in excess Gurken protein, supporting the model that *gurken* is a target of repression. Two modifiers were mapped to individual genes. One is *Lk6*, which encodes a protein kinase predicted to regulate the rate-limiting initiation factor eIF4E. The second is *Delta*. The interaction between *arrest* and *Delta* mimics the phenotype of homozygous *Delta* mutants, suggesting that *arrest* could positively control *Delta* activity. Indeed, *arrest* mutants have significantly reduced levels of Delta protein at the interface of germline and follicle cells.

INTRODUCTION

Translational control has long been recognized as an important regulatory process in the early stages of animal development. Classical studies focused on the dramatic change in translation activity that occurred after fertilization in amphibians (Davidson, 1986). More recent discoveries revealed transcript-specific forms of regulation, such as the coordinated activation of large classes of mRNAs by interaction of CPE binding protein with the Cytoplasmic Polyadenylation Element (CPE) and subsequent extension of the poly(A) tail (Mendez and Richter, 2001). Control events that may be specific for small numbers of mRNAs have emerged from the analysis of body patterning in Drosophila, where the transcripts encoding determinants that define the dorsoventral and anteroposterior axes of the egg are subject to elaborate programs of localization and translational regulation (Lipshitz and Smibert, 2000). For the *bicoid* mRNA, which encodes the anterior determinant, translation appears to be activated as a consequence of poly(A) tail extension shortly after fertilization (Sallés et al., 1994). The *oskar* (*osk*) and *gurken* (*grk*) mRNAs, which encode proteins whose positions specify posterior and dorsal fates, respectively, are under more complex forms of control. Transcription of *osk* mRNA begins very early in oogenesis, but Osk protein does not appear at high levels until mid oogenesis when the *osk* mRNA becomes localized to the posterior pole of the oocyte (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). The translational repression of *osk* mRNA prior to its localization is achieved in part through the action of Bruno (Bru), a protein that binds to regulatory sequences, BREs, in the *osk* 3' UTR (Kim-Ha et al., 1995; Webster et al., 1997). Bru also appears to regulate the *grk* mRNA, as Bru can bind to *grk* mRNA and overexpression of a *bru* cDNA leads to dorsoventral patterning defects consistent with a reduction in Grk activity (Filardo and

Ephrussi, 2003; Kim-Ha et al., 1995; Norvell et al., 1999). At present the mechanism by which Bru represses translation is only poorly understood.

Bru is encoded by the *arrest* (*aret*) gene (Schüpbach and Wieschaus, 1991; Webster et al., 1997). Bru protein was characterized and named before the discovery that it corresponds to *aret*, and is widely known by the name Bru. In this paper we refer to the gene and mutants as *aret*, and the gene products as Bru. The phenotype of *aret* mutants suggests that Bru is likely to regulate other mRNAs in addition to the known targets, since some aspects of the phenotype are not readily attributed to misregulation of either *osk* or *grk*. During wild type oogenesis cysts of 16 germline cells, which remain interconnected because of incomplete cytokinesis, are formed in the germarium. Individual cysts bud off and become enveloped by a layer of somatic follicle cells to create an egg chamber. One germline cell in each cyst becomes specified as the oocyte, while the remaining 15 become nurse cells (Spradling, 1993). In strong *aret* mutants, such as $aret^{QB}$, the cysts contain variable numbers of germline cells that complete cytokinesis and fail to differentiate and specify an oocyte (Parisi et al., 2001; Schüpbach and Wieschaus, 1991). Overexpression of *osk* or *grk* mutants does not cause such a phenotype (Ghiglione et al., 2002; Neuman-Silberberg and Schupbach, 1994; Smith et al., 1992), suggesting that *aret* regulates expression of one or more other mRNAs.

Some alleles of *aret* have weaker phenotypes that also reveal the pleiotropic character of the gene (Schüpbach and Wieschaus, 1991; Webster et al., 1997). In intermediate allelic combinations, such as $aret^{QB}/aret^{PD}$ or $aret^{QB}/aret^{PA}$, morphologically normal egg chambers form and an oocyte is specified, but after stage 6 or 7 the egg chambers become necrotic. Again, overexpression of either *osk* or *grk* does not lead to this phenotype. Females of the weakest allelic combinations, such as $aret^{PD}/aret^{PA}$, produce eggs that can be fertilized and develop to a late stage of

embryogenesis. Many of the embryos have irregular anteroposterior patterning defects revealed by alterations of the ventral denticle belts: some individual segments are missing or adjacent segments are partially fused. Although overexpression of *osk* or *grk* does cause embryonic patterning defects, these defects are very specific and consistent, and are unlike those of the *aret* mutants.

One genetic approach that could be useful in learning more about the function of Bru is an interaction screen, in which mutants are tested for their ability to modify *aret*$^{-}$ phenotypes. This approach has been used with considerable success to identify loss-of-function mutants that dominantly modify an existing phenotype (Simon et al., 1991). An interaction screen offers the possibility of providing insights into two of the main questions about the function of Bru. How does Bru repress translation? And what additional mRNAs are regulated by Bru? A reduction in the dosage of a gene that acts in the same process as Bru could enhance or suppress the *aret* phenotype. Similarly, a reduction in the level of an mRNA normally repressed by Bru could suppress the *aret* phenotype. Here we describe the results of such a screen.

RESULTS

To identify genes that interact with *aret* we tested third chromosome deficiency mutants for the ability to dominantly suppress the ovarian *aret* phenotype. The mutants are from the 'deficiency kit' maintained by the Bloomington Stock Center, and the subset that could be used (those not requiring the presence of a duplication) cover about 74% of the chromosome. To streamline the crossing scheme the $aret^{PD}$ chromosome was marked with the dominant eye marker, S^{1}, such that flies of the desired genotype could be identified despite the absence of second chromosome markers or balancer chromosomes in the deficiency stocks (see Fig. 2.1). Serendipitously, the S^{1} mutation proved to dominantly enhance the *aret* phenotype, thus identifying an initial modifier mutant.

For each genotype the ovaries from multiple females were dissected and fixed, stained with DAPI to highlight nuclei, and examined by fluorescence microscopy. Wild type or $aret^{PD}$ heterozygous females have ovaries with all stages of oogenesis represented (Fig. 2.2A). In contrast, oogenesis in S^{1} $aret^{PD}$/*Df(2L)esc-P2-0* females is arrested at the germarial stage (Fig. 2.2B). The vast majority of the deficiency mutants have no consistent effect on the S^{1} $aret^{PD}$/*Df(2L)esc-P2-0* oogenesis phenotype (Fig. 2.2C; Table 1), thus the starting phenotype is not overly sensitive to variations in genetic background. Only four deficiencies elicited a clear dominant alteration of the starting phenotype, either delaying the arrest (Fig. 2.2E) or increasing the number of germ cells in undifferentiated germaria (Fig. 2.2D). These, as well as S^{1}, were examined in detail. Not surprisingly, in no case was suppression of the *aret* phenotype complete. Instead, the arrest of oogenesis was extended to a later stage of development, but not to the point of egg laying.

Star^{1} interacts with *aret*

Ovaries of *aret*^{PD}/*Df(2L)esc-P2-0* females have an intermediate *aret* phenotype, in which oogenesis proceeds as far as stage 7 before the egg chambers degenerate (Fig. 2.3A). When S^{1} is also present, in S^{1} *aret*^{PD}/*Df(2L)esc-P2-0* ovaries, the arrest of oogenesis is advanced to the germarial stage (Fig. 2.3B). S^{1} has no dominant ovarian phenotype by itself (data not shown), and is thus acting as a dominant enhancer of the starting phenotype. This genetic interaction could be between *S* and *aret*, which is homozygous mutant in the affected flies, or between *S* and any of the genes made hemizygous by *Df(2L)esc-P2-0*. To distinguish between these possibilities we asked if S^{1} enhances the phenotype of flies in which only *aret* is mutant. The *aret*^{PD}/*aret*^{PA} allelic combination has a weak phenotype (Fig. 2.3C), with many egg chambers producing mature oocytes that can be fertilized and progress through much of embryogenesis. When S^{1} is also present, the phenotype becomes more severe and oogenesis arrests at stage 6 or 7 (Fig. 2.3D). The phenotype of *aret*^{PD}/*aret*^{QB}, in which an oocyte is specified but oogenesis arrests at stage 6/7 (Fig. 2.3E), is similarly enhanced by S^{1}. The arrest occurs earlier, at about stage 3 (Fig. 2.3F), and no oocyte is specified (data not shown). We conclude that S^{1} is interacting with *aret*. This interaction does not appear to reflect Bru-dependent regulation of *S* mRNA translation, as no changes in S protein levels were detected (data not shown). The *aret*/S^{1} interaction is persistent during oogenesis, occurring at different stages. Thus either *aret* and *S* interact in an ongoing process, or the severity of the defect in some initial process influences later events.

A genetic interaction between *S* and *aret* is not surprising, since both have been implicated in expression or activity of *grk*. Grk is a transforming growth factor (TGF) alpha-like protein that is expressed as a membrane-bound form in the germline (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1996; Serano et al.,

1995). After proteolytic cleavage separates intra- and extracellular domains, the latter activates epidermal growth factor receptor (Egfr) in the overlying follicle cells (Ghiglione et al., 2002; Ray and Schupbach, 1996). *S* is required for the activity of Grk and appears to act in post-cleavage trafficking or secretion of the protein (Ghiglione et al., 2002). Overexpression of Bru reduces the level of *grk* activity and the amount of localized Grk protein (Filardo and Ephrussi, 2003). Thus the combined effects of reduction of both *aret* and *S* activity could well affect the level of *grk* activity. To determine if the amount or distribution of Grk protein is altered in S^1 $aret^{PD}/aret^{PA}$ ovaries, we monitored the protein in whole mount preparations by immunofluorescence. In wild type ovaries Grk protein accumulates in the oocyte during early stages (Fig. 2.4A), and then becomes restricted to an anterodorsal region over the oocyte nucleus in stages 8-10. A similar Grk accumulation is observed in *aret* mutant ovaries (Fig. 2.4B). When the S^1 mutation is also present, in S^1 $aret^{PD}/aret^{PA}$ ovaries, Grk protein can now be detected at low levels in nurse cells (Fig. 2.4C). In some preparations this nurse cell staining is concentrated at cell boundaries (Fig. 2.4D), as if the protein is membrane-associated.

Lk6, which encodes a protein kinase predicted to phosphorylate eIF4E, interacts with *aret*

Df(3R)M-Kx1, which removes parts of cytological intervals 86 and 87, dominantly suppresses the S^1 $aret^{PD}$/*Df(2L)esc-P2-0* phenotype (Fig. 2.2E). The interaction is with *aret*, rather than *S* or *Df(2L)esc-P2-0*, since *Df(3R)M-Kx1* also suppresses $aret^{PD}/aret^{QB}$ (Fig. 2.5A and B) and $aret^{PA}/aret^{QB}$ (data not shown). The interacting gene was identified by testing smaller deficiencies and P element insertion mutants for the interaction (Fig. 2.5C and data not shown). The P element mutant *EP(3)0886*, which is inserted 5' to the *Lk6* gene (Huang and Rubin, 2000; Kidd and Raff, 1997), suppresses the phenotype of $aret^{PD}/aret^{QB}$ (Fig. 2.5C) and of $aret^{PD}$/*Df(2L)esc-*

P2-0 (data not shown). EP P elements, such as *EP(3)0886*, contain a GAL4-inducible promoter that, in conjunction with a source of GAL4, directs expression of the endogenous gene located proximal to the P element (Rorth et al., 1998), in this case *Lk6*. The suppression of the *aret* phenotype occurs in the absence of GAL4, consistent with the notion that the EP insertion by itself reduces *Lk6* expression. When a germline source of GAL4 is provided, *EP(3)0886* enhances the $aret^{PD}/aret^{QB}$ phenotype (Fig. 2.5D). The complementary suppression and enhancement from the same P element demonstrates that *Lk6* interacts with *aret*. This interaction could reflect Bru-dependent control of *Lk6* mRNA translation. This interpretation is unlikely, as Lk6 protein levels appear similar in wild type and *aret* mutants (data not shown).

The effect of the *Lk6* mutation on the *aret* oogenesis arrest phenotype persists throughout oogenesis, as different *aret* allelic combinations that arrest oogenesis at different stages all show suppression. The early arrest of $aret^{QB}/aret^{QB}$ at stage 2 is extended to stage 3 or 4 when *EP(3)0886* is present, and the stage 6/7 arrest of $aret^{PD}/aret^{QB}$ is extended to stage 9. Furthermore, the low hatch rate of embryos from $aret^{PA}/aret^{PD}$ mothers (3% n=300) is elevated (14% n=300) when the mothers also carry one copy of *EP(3)0886*. The suppression of *aret* mutants by reduction of *Lk6* is paralleled by enhancement of the phenotype when *Lk6* is overexpressed. In the $aret^{PD}/aret^{QB}$ combination the arrest was shifted from stage 6/7 to stage 3, and with the $aret^{PA}/aret^{PD}$ combination, which normally allows the completion of oogenesis, oogenesis was arrested at stage 9/10.

In addition to the oogenesis arrest phenotype, strong *aret* mutants can have cysts with greater than 16 germline cells (Parisi et al., 2001; Schüpbach and Wieschaus, 1991). This aspect of *aret* function does not appear to interact with *Lk6*: neither the deficiency

that removes *Lk6*, *Df(3R)M-Kx1*, nor *EP(3)0886* suppresses the extra germline cells phenotype (data not shown).

The Lk6 protein is predicted to phosphorylate and thus enhance activity of translation initiation factor eIF4E (Lachance et al., 2002), and could act through that pathway in suppressing the *aret* phenotype. Specifically, a global reduction in translation efficiency from reduced eIF4E activity could offset the enhanced translation of an mRNA negatively regulated by *aret*. To evaluate this possibility we asked if expression of a constitutively activated eIF4E, eIF4E S251D, in which the serine predicted to be phosphorylated by Lk6 is mutated to a negatively charged amino acid (Lachance et al., 2002), would reverse the suppression caused by the *Lk6* mutant. Females of the genotype $aret^{QB}/aret^{PD}$*; EP(3)0886/+* show the same timing of arrest - at stage 9 - as females of the genotype $aret^{QB}/aret^{PD}$ *P[eIF4E S251D]; EP(3)0886/+*. This result suggests that expression of the constitutively activated eIF4E has no effect on the suppression of the *aret* phenotype by the *Lk6* mutant. However, the *P[eIF4E S251D]* transgene can itself suppress the $aret^{QB}/aret^{PD}$ phenotype, and delay arrest until stage 9. Thus either enhanced activity of eIF4E, provided by the *P[eIF4E S251D]* transgene, or a predicted reduction of eIF4E activity, from the *Lk6* mutant, has the same effect on the *aret* phenotype, and interpretation of the suppression observed in the $aret^{QB}/aret^{PD}$ *P[eIF4E S251D]; EP(3)0886/+* females is not simple.

Additional Dfs dominantly suppress the timing of oogenesis arrest

Only two other third chromosome deficiency mutants suppressed the timing of oogenesis arrest of S^{1} $aret^{PD}$*/Df(2L)esc-P2-0* females. One of these, *Df(3R)mbc-R1*, also suppressed the arrest phenotype of $aret^{QB}/aret^{QB}$, showing that it is acting on *aret*. Just as for *Lk6*-mediated suppression, there was no effect on the occurrence of cysts with

greater than 16 germline cells. We have not been able to identify a point mutant from within the region uncovered by this deficiency that displays a similar interaction.

The other suppressing deficiency, *Df(3R)vin5*, subsequently failed to suppress either $aret^{QB}$ homozygotes or $aret^{PA}/aret^{QB}$ females. This deficiency presumably interacts with S^{1}, with *Df(2L)esc-P2-0*, or with a combination of the mutations on the input chromosomes, and was not characterized further.

***Delta* and *aret* interact**

Females of genotype S^{1} $aret^{PD}$*/Df(2L)esc-P2-0; Df(3R)Dl-BX12*/+ have ovaries in which no cysts with the usual 16 germ cells form. Instead, in each ovariole a single layer of follicle cells surround a large number of undifferentiated germ cells (Fig. 2.2D). The interacting genes responsible for the phenotype are *Delta* (*Dl*) and *aret* ; females homozygous or transheterozygous for *aret* alleles and heterozygous for Dl^{9P}, an amorphic allele, show a similar phenotype. Females homozygous for the strong $aret^{QB}$ allele, in which cysts form but no oocyte is specified, and heterozygous for Dl^{9P}, have an ovarian phenotype similar to that observed with *Df(3R)Dl-BX12* in the screen (Fig. 2.6). In $aret^{QB}/aret^{QB}$ ovaries, no oocyte is specified (Fig. 2.6A) and individual cysts sometimes have greater than 16 germline cells (Fig. 2.6A'). When also heterozygous for Dl^{9P}, almost all ovarioles now consist of the germarium and a single large egg chamber with many germline cells (the egg chamber is small in rare exceptions). This egg chamber could arise from fusion of initially separate egg chambers, or from overproliferation of the germ line. It is often possible to distinguish between these options, since the cell divisions that produce the germline cells have incomplete cytokinesis. Consequently, each daughter cell remains connected to its parent by a ring canal, and a branched organelle, the fusome, extends through the ring canals (Fig. 2.6C). The number of cell divisions within a cyst can be determined by counting the number of

ring canals of the oocyte. However, in the strong *aret* mutants cytokinesis is complete, there are no ring canals, and the fusome appears as dots (Fig. 2.6D). In the single large egg chamber of $aret^{QB}/aret^{QB}$; $Dl^{9P}/+$ ovarioles, the fusome also appears as dots (Fig. 2.6E), and so we unable to determine how these egg chambers form. The *Dl* mutation could be enhancing the frequency and severity of the germline overproliferation phenotype of $aret^{QB}$. Alternatively, the *aret* mutations could be enhancing the egg chamber fusion phenotype of *Dl* mutants (Lopez-Schier and St Johnston, 2001).

A weaker combination of *aret* alleles also interacts with *Dl9P*. In $aret^{PD}/aret^{QB}$ ovaries, oogenesis proceeds as far as stage 6/7 (Fig. 2.7D). In $aret^{PD}/aret^{QB}$; $Dl^{9P}/+$ ovaries most egg chambers have greater than 16 germline cells (Fig. 2.7A-C), a situation that can again occur by overproliferation within a single egg chamber or fusion of multiple egg chambers. This genotype produces three classes of abnormal egg chambers, which are present in roughly equal numbers. In one class, a large number of germline cells of roughly equal size are enveloped by a single epithelium of follicle cells (Fig. 2.7A). This phenotype is similar to that seen when using the stronger *aret* alleles, including the absence of an oocyte. The remaining two classes clearly arise from fusion. They both display partial fusion, with individual cysts failing to separate from one another, though they differ in the nature of the fusions.

In wild type and $aret^{PD}/aret^{QB}$ ovaries adjacent egg chambers are connected by several stalk cells positioned between the anterior polar cell of one egg chamber and the posterior polar cell of the next (Fig. 2.7D and E). For the partially fused egg chambers of $aret^{PD}/aret^{QB}$; $Dl^{9P}/+$ flies, no stalk cells can be detected and the follicle cell layers of different egg chambers remain in intimate contact with one another (Fig. 2.7C'). Some maintain a well-defined linear organization within individual ovarioles, and adjacent egg chambers are fused with each other at their anterior and posterior boundaries (Fig. 2.7C);

we refer to this class as A/P fusions. The other class of partially fused egg chambers – called random fusions - reside in ovarioles in which the normal beads-on-a-string organization is absent. Egg chambers are positioned irregularly and can be closely apposed to multiple different egg chambers on lateral as well as anterior and posterior surfaces (Fig. 2.7B). For both classes of partial fusion each cyst has an oocyte as determined by the presence of a single cell with a high concentration of the oocyte marker Orb (data not shown), although specific Orb staining is lost at the later stages when the cysts begin to degenerate. In the A/P fusions the oocyte is clearly at the posterior of the cyst. Defining the position of the oocyte in the random fusions is somewhat subjective, given the absence of an tandem arrangement of cysts, but in all cases the oocyte is either lateral or posterior relative to the overall polarity of the ovariole (data not shown).

The parital fusion phenotypes of $aret^{PD}/aret^{QB}; Dl^{9P}/+$ are very similar to those of *Dl/Dl* germline clones (see Discussion). A simple interpretation of this similarity is that Dl activity is reduced in *aret* mutants, through a reduction in the synthesis or presentation of Dl protein. To test this prediction we examined Dl protein in wild type and $aret^{PD}/aret^{QB}$ ovaries. Dl normally accumulates at the highest levels in the membranes separating the follicular epithelium from the nurse cells and oocyte, with a lower level of cytoplasmic staining (Fig. 2.8A). In the *aret* mutant the level of membrane-associated Dl is clearly reduced, although the cytoplasmic staining is not visibly different (Fig. 2.8B). Because the role of Dl is in signaling from the germline cell to the follicle cells, the protein at the junction between these cells is expected to be the active form. Thus *aret* does appear to have a positive role, probably indirect, in promotion of *Dl* activity.

In the third class of abnormal egg chambers a large number of germline cells of roughly equal size are enveloped by a single epithelium of follicle cells (Fig. 2.7A). This phenotype is similar to that seen when using the stronger *aret* alleles, including the absence of an oocyte.

DISCUSSION

We performed a screen of third chromosome deficiencies for dominant modifiers of *aret* mutants. About three quarters of the third chromosome was screened, corresponding to roughly 30% of the genome. Only four deficiencies dominantly modified the *aret* mutant phenotype, suggesting that the total number of genes in the genome with this property is small. For two of the four deficiencies we were able to identify the gene responsible for the interaction, and we fortuitously discovered a third interacting gene while preparing for the screen. We anticipated that two different types of modifiers might be detected by the screen: those in genes that act in the same process as Bru, and those in genes that are themselves regulated by Bru, or act in a process in which a limiting component is regulated by Bru. Characterization of the interacting genes suggests that we recovered examples of each type of modifier.

Interaction of *aret* and *S*

Bru has been proposed to translationally regulate *grk* mRNA. The supporting evidence includes (i) binding of Bru to *grk* mRNA in vitro and indirect evidence of binding in vivo (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995; Norvell et al., 1999), (ii) rare dorsoventral patterning defects as a consequence of overexpression of Bru, and enhancement of this phenotype by reduction of *grk* gene dosage, and (iii) evidence that localized Grk is present at reduced levels when Bru is overexpressed, although unlocalized Grk appears more abundant (Filardo and Ephrussi, 2003). However, there

has been no evidence of excess Grk protein in *aret* mutants. *S* is required for *grk* activity, and it acts post-translationally in either trafficking or secretion of Grk protein (Ghiglione et al., 2002). We found that when flies were both homozygous for *aret* and heterozygous for S^1 they accumulated Grk protein in nurse cells, while ectopic accumulation could not be detected in either *aret* mutants or S^1 heterozygotes alone. This synthetic effect on Grk protein accumulation is simple to rationalize. In *aret* mutants Grk protein is excessively translated, but an *S*-dependent delivery step could efficiently clear the protein from the nurse cells. When *S* activity is reduced, a detectable level of Grk remains in the nurse cells. The distribution of the ectopic Grk, in both cytoplasm and at the nurse cell boundaries, could correspond to the sites where the protein might stall during delivery. The actual site of S action is not known, and two different sites of S concentration, in endoplasmic reticulum or on the plasma membrane, have been reported (Ghiglione et al., 2002; Pickup and Banerjee, 1999). Although this explanation has some appeal, it is important to note that none of the evidence firmly establishes a role for Bru in translational repression of *grk* mRNA, and it remains possible that Bru could, for example, influence the site of translation rather than its efficiency.

Although the combination of S^1 and *aret* mutations does affect Grk expression or distribution, there are no precedents that clearly demonstrate how excess or ectopic Grk would enhance the oogenesis arrest phenotype of *aret* mutants. Thus the explanation for the enhancement remains unknown, and could involve the effects on *grk* or on other genes that are subject to regulation by Bru.

Is there a link between Bru and initiation of translation?

The eIF4E protein binds to the cap at the 5' end of mRNAs. It is a rate-limiting component of translational initiation, and its activity is under tight control (Gingras et al., 1999). One form of regulation is phosphorylation, which is thought to control the mRNA

cap-binding activity of eIF4E (Marcotrigiano et al., 1997; Raught et al., 2000). Several lines of correlative evidence suggest that this phosphorylation is important for cell proliferation (Bonneau and Sonenberg, 1987; Raught et al., 2000), and mutation of the Drosophila eIF4E to prevent phosphorylation results in reduced viability and poor growth (Lachance et al., 2002).

A transgene expressing a mutant and constitutively activated version of eIF4E, in which the regulatory phosphorylation is mimicked by a amino acid change, can suppress the *aret* phenotype. This result raises the possibility that Bru has a positive role in initiation of translation. Specifically, in the *aret* mutant one or more target mRNAs that require Bru for activation of translation may be underexpressed, and increasing translation suppresses this defect.

However, the *aret* mutant phenotype is also suppressed by a mutation of *Lk6*, and enhanced by overexpression of *Lk6*. Lk6 is the Drosophila protein most closely related to mammalian mitogen-activated protein kinase-interacting protein kinase 1 (MNK1), which phosphorylates translation initiation factor eIF4E after activation by either the p44/42 or p38 MAPKs (Fukunaga and Hunter, 1997; Lachance et al., 2002; Waskiewicz et al., 1997). Thus mutation of *Lk6* might be expected to reduce eIF4E phosphorylation and thereby decrease translational capacity. By this view the suppression of the *aret* phenotype would be consistent with an interaction between eIF4E and Bru that involves the known function of Bru in translational repression. In favor of this notion (Nakamura et al., 2004) have recently shown that Bru physically interacts with Cup, an eIF4E binding protein that is required for repression of *osk* mRNA translation. To explore this possibility further we asked if suppression of the *aret* phenotype by *EP(3)0886* was accompanied by a change in the levels of Osk or Grk proteins, or if homozygous *EP(3)0886* females have abnormal amounts of either protein. No change was seen in

either case (data not shown). Thus we do not know if the Lk6 mutation impacts the function of *aret* in repression of *osk* or *grk* mRNAs.

Given the similar consequences on the *aret* phenotype of the constitutively active eIF4E and the mutant predicted to reduce eIF4E activity, the simplest explanation is that *Lk6* may affect *aret* function by a means other than phosphorylation of eIF4E. Suppression of the *aret* phenotype by the mutant eIF4E clearly suggests a link between Bru and the initiation of translation, although this need not be direct.

Interaction of *aret* and *Dl*

The combination of $aret^{PD}/aret^{QB}$ with $Dl^{9P}/+$ produces a variety of ovarian defects, complicating interpretation of the phenotype. Nevertheless, one striking feature is the similarity of many of the defects to those seen when *Dl* activity is largely or completely eliminated, suggesting that the *aret* mutations are enhancing the *Dl* phenotype. *Dl* is a component of the *Notch/Dl* signaling pathway, which acts in many signaling events in a wide range of cell types (Artavanis-Tsakonas et al., 1999). In the ovary *Dl* is required in the germline cells for control of differentiation and proliferation of the somatic follicle cells, and for setting up anteroposterior polarity (Lopez-Schier and St Johnston, 2001; Torres et al., 2003). The earliest and, at least initially, most dramatic consequence of loss of *Dl* activity is the fusion of cysts - the phenotype most apparent in the $aret^{PD}/aret^{QB}$; $Dl^{9P}/+$ ovaries.

Large germ line clones of strong *Dl* mutant alleles cause a complete fusion of egg chambers into a single egg chamber with multiple cysts, reminiscent of the complete fusions described here. Smaller clones retain a more regular ovariole organization. Individual egg chambers with *Dl* germ line clones often fuse with the adjacent anterior wild type egg chamber. Fusion can be incomplete, resulting in a double layer of follicle cells that separate the egg chambers, much as observed for the A/P partial fusions we

report. However, the similarities are not perfect. For example, *Dl* mutant clones upregulate FasIII in the follicular epithelium (Lopez-Schier and St Johnston, 2001), but $aret^{PD}/aret^{QB}$; Dl^{9P}/+ egg chambers do not (data not shown). Other features of the *Dl* mutant phenotype, such as the defects in anteroposterior polarity (Torres et al., 2003), are difficult to detect in the $aret^{PD}/aret^{QB}$; Dl^{9P}/+ ovaries, because of their arrest of oogenesis. The lack of perfect correspondence between the *Dl* germ line clones and the $aret^{PD}/aret^{QB}$; Dl^{9P}/+ ovaries is not surprising for several reasons. First, there is substantial phenotypic variation even among the *Dl* germ line clones, if both large and small clones are considered. Second, the clones are homozygous for Dl^{-}, while in the *aret* mutant background one wild type copy of *Dl* remains. Third, the *Dl*-like defects in $aret^{PD}/aret^{QB}$; Dl^{9P}/+ ovaries are superimposed on the *aret* mutant phenotype.

The simplest interpretation of our results is that the *aret* mutations are reducing the activity of the *N*/*Dl* signaling pathway, which in combination with mutation of one copy of *Dl* leads to phenotypes similar to those resulting from loss of *Dl*. This model is fully supported by the finding that in *aret* mutants the amount of Dl protein concentrated at the border between germline cells and follicle cells is reduced. What remains unclear is how this reduction occurs. Assuming that Bru is acting as a translational repressor, in the *aret* mutant the target protein should be present at elevated levels. By this model the target should be a gene that normally has a negative effect on *Dl* expression or delivery to the membrane. Alternatively, Bru could also have a role in translational activation, in which case *Dl* could be a direct target. This seems quite unlikely, as the *Dl* 3' UTR lacks any recognizable BREs, the sequences to which Bru is known to bind. Nevertheless, a role for Bru in translational activation is possible, and the target could normally have a positive effect on provision of *Dl* activity.

MATERIALS AND METHODS

Fly stocks: *aret* mutants (Schüpbach and Wieschaus, 1991) were from Trudi Schupbach. Deficiency mutants, P element insertion mutants, and the *Star*^{1} (*S*^{1}) allele were obtained from the Bloomington Stock Center. *P[eIF4E S251D]* was from Paul Lasko. *nosGAL4VP16* (Van Doren et al., 1998) was from Ruth Lehmann. *matα4-GAL4-VP16#6* was obtained by jumping the P element in *matα4-GAL4-VP16 V32a* (Martin and St Johnston, 2003), obtained from Daniel St Johnston. The *S*^{1} *aret*^{PD} and *aret*^{PD} *P[eIF4E S251D]* chromosomes were constructed by recombination using standard genetic methods.

Deficiency screen: The cross scheme shown in Fig. 2.1 was used to generate adults of the desired genotypes. After 3-4 days in well-yeasted vials, females were dissected and the ovaries fixed in 4% formaldehyde in PBS for 20-30 minutes. After rinsing several times in PBT (PBS plus 0.1% Triton X-100), the ovaries were mounted in Vectastain (Vector Labs) containing DAPI to label nuclei. Ovaries were examined using a Nikon epifluoresence microscope.

Antibody staining: Ovaries were fixed as described above, blocked in PBT plus 5% goat serum, and incubated overnight in PBT plus 1% goat serum and the primary antibody. After several washes in PBT plus 1% goat serum, secondary antibodies were added for 2 hours, followed by several washes in PBT. In some cases DNA was counterstained with ToPro-3 (Molecular Probes) diluted 1/1000. Samples were mounted in Vectastain and imaged by confocal microscopy with a Lecia TCS-SP. Primary antibodies, all from Developmental Studies Hybridoma Bank unless otherwise noted, were used at the following dilutions: mouse anti-Grk, 1/10; rat anti-Star (Uptal Banerjee), 1/10; mouse anti-Orb, 1/20; mouse anti-Hts 1B1, 1/10; mouse anti-FasIII, 1/10; rabbit

anti-Lk6 (Jordan Raff), 1/500; mouse anti-Dl EC, 1/100; rabbit anti-Vas (Paul Lasko), 1/2000.

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TABLE

Table 1. Deficiencies used in the screen.

Genotype	Breakpoints	Modification
Df(3L)emc-E12	061A;061D03	
Df(3L)Ar14-8	061C05-08;062A08	
Df(3L)Aprt-32	062B01;062E03	
Df(3L)R-G7	062B08-09;062F02-05	
Df(3L)M21	062F;063D	
Df(3L)HR119	063C02;063F07	
Df(3L)GN34	063E06-09;064A08-09	
Df(3L)GN24	063F06-07;064C13-15	
Df(3L)ZN47	064C;065C	
Df(3L)XDI98	065A02;065E01	
Df(3L)BSC27	065D04-05;065E04-06	
Df(3L)pbl-X1	065F03;066B10	
Df(3L)BSC13	066B12-C01;066D02-04	
Df(3L)h-i22	066D10-11;066E01-02	
Df(3L)Scf-R6	066E01-06;066F01-06	
Df(3L)29A6	066F05;067B01	
Df(3L)AC1	067A02;067D07-13 or 067A05;067D09-13	
Df(3L)BSC14	067E03-07;068A02-06	
Df(3L)lxd6	067E05-07;068B02-04	
Df(3L)vin2	067F02-03;068D06	

Df(3L)vin5	068A02-03;069A01-03	+
Df(3L)iro-2	069B01-05;069D01-06	
Df(3L)E44	069D02;069E03-05	
In(3LR)C190[L]	069F03-04;070C03-04	
Df(3L)fz-GF3b	070C01-02;070D04-05	
Df(3L)fz-M21	070D02-03;071E04-05	
Df(3L)BK10	071C;071F	
Df(3L)brm11	071F01-04;072D01-10	
Df(3L)st-f13	072C01-D01;073A03-04	
Df(3L)81k19	073A03;074F	
Df(3L)W10	075A06-07;075C01-02	
Df(3L)Cat	075B08;075F01	
Df(3L)XS2182	076B;076F	
Df(3L)XS543	076B;077A	
Df(3L)kto2	076B01-02;076D05	
Df(3L)XS533	076B04;077B	
Df(3L)XS572	076B06;077C01	
Df(3L)rdgC-co2	077A01;077D01	
Df(3L)ri-79c	077B-C;077F-78A	
Df(3L)ME107	077F03;078C08-09	
Df(3L)31A	078A;078E, 078D;079B	
Df(3L)Pc-2q	078C05-06;078E03-079A01	
Df(3L)Ten-m-AL29	079C01-03;079E03-08	
Df(3L)HD1	079D03-E01;079F03-06	
Df(3L)Delta1AK	079E05-F01;079F02-06	

Df(3R)ME15	081F03-06;082F05-07	
Df(3R)3-4	082F03-04;082F10-11	
Df(3R)e1025-14	082F08-10;083A01-03	
Df(3R)Scr	084A01-02;084B01-02	
Df(3R)Antp17	084B01-02;084D11-12 or A06,D14	
Df(3R)p712	084D04-06;085B06	
Df(3R)p-XT103	085A02;085C01-02	
Df(3R)BSC24	085C04-09;085D12-14	
Df(3R)by10	085D08-12;085E07-F01	
Df(3R)by62	085D11-14;085F06	
Df(3R)M-Kx1	086C01;087B01-05	+
Df(3R)ry615	087B11-13;087E08-11	
Df(3R)ea	088E07-13;089A01	
Df(3R)sbd105	088F09-89A01;089B09-10	
Df(3R)P115	089B07-08;089E07-08;020	
Df(3R)DG2	089E01-F04;091B01- B02	
Df(3R)C4	089E03-04;090A01-07	
Df(3R)Cha7	090F01-F04;091F05	
Df(3R)Dl-BX12	091F01-02;092D03-06	+
Df(3R)H-B79	092B03;092F13	
Df(3R)e-N19	093B;094	
Df(3R)e-R1	093B06-07;093D02	
Df(3R)mbc-R1	095A05-07;095D06-11	+
Df(3R)crb-F89-4	095D07-D11;095F15	
Df(3R)crb87-5	095F07;096A17-18	

Df(3R)slo[8]	096A02-07;096D02- 04
Df(3R)Espl3	096F01;097B01
Df(3R)Tl-P	097A;098A01-02
Df(3R)D605	097E03;098A05
Df(3R)3450	098E03;099A06-08
Df(3R)L127	099B05-06;099E04-F01
Df(3R)B81	099C08;100F05

FIGURES

Figure 2.1. Cross scheme to test deficiency mutants for modification of the *aret* mutant phenotype.

The inclusion of S^1, which has a dominant visible rough eye phenotype, on the $aret^{PD}$ chromosome allows us to identify the $S^1\ aret^{PD}/Df(2L)esc\text{-}P2\text{-}0$ individuals without requiring that the third chromosome mutants be balanced or marked on their second chromosomes.

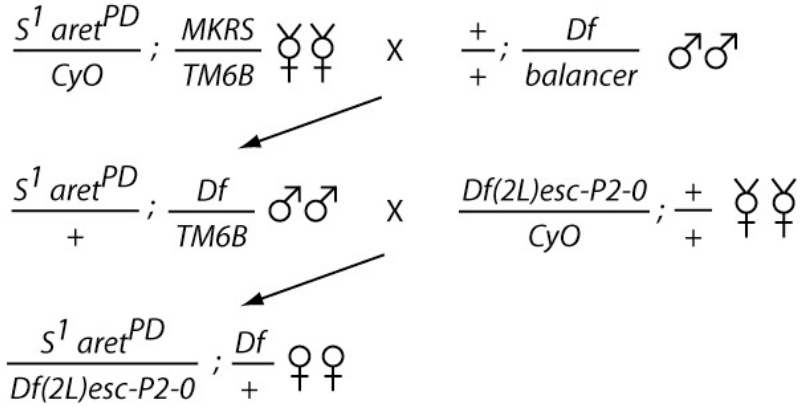


Figure 2.2. Phenotypes scored in the screen.

All panels are micrographs of ovarioles stained with DAPI (D) or ToPro-3 (all other panels) to highlight nuclei.

A. Part of a wild type ovariole, including the germarium at left and several egg chambers of increasing maturity.

B. Oogenesis is arrested at a very early stage in S^{1} *aret*^{PD}*/Df(2R)esc-P2-0* ovaries and the two ovarioles shown fail to bud off individual egg chambers. Instead, multiple undifferentiated germ cells appear in the severely truncated ovarioles.

C. An example of a third chromosome deficiency, *Df(3L)pbl-X1*, that has no dominant effect on the starting phenotype.

D. *Df(3R)Dl-BX12* dominantly alters the starting phenotype, such that the germarium is greatly expanded to produce a large volume of germ cells surrounded by a layer of follicle cells.

E. Partial dominant suppression of the S^{1} *aret*^{PD}*/Df(2R)esc-P2-0* phenotype by *Df(3R)M-Kx1*. Egg chambers bud off from the germarium, although they are abnormal. Each chamber has greater than the usual number of 16 germ cells, and no oocyte is specified.

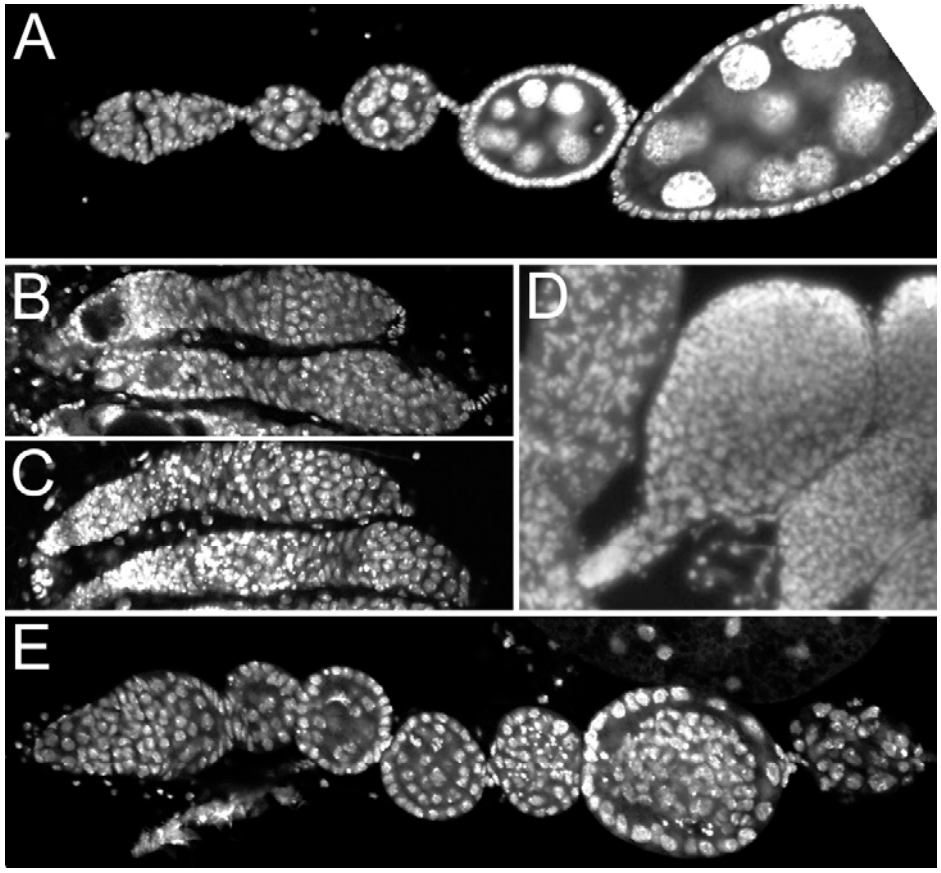

A
B
C
D
E

Figure 2.3. *Star* interacts with *aret*

The phenotypes of three *aret* allelic combinations of different strength are all dominantly enhanced by S^1. In each case the *aret* phenotype becomes more severe, largely mimicking the effect of using a stronger *aret* allelic combination.

A. $aret^{PD}$/*Df(2R)esc-P2-0.*

B. S^1 $aret^{PD}$/*Df(2R)esc-P2-0.*

C. $aret^{PD}$ /$aret^{PA}$.

D. S^1 $aret^{PD}$/$aret^{PA}$.

E. $aret^{PD}$/$aret^{QB}$.

F. S^1 $aret^{PD}$/$aret^{QB}$.

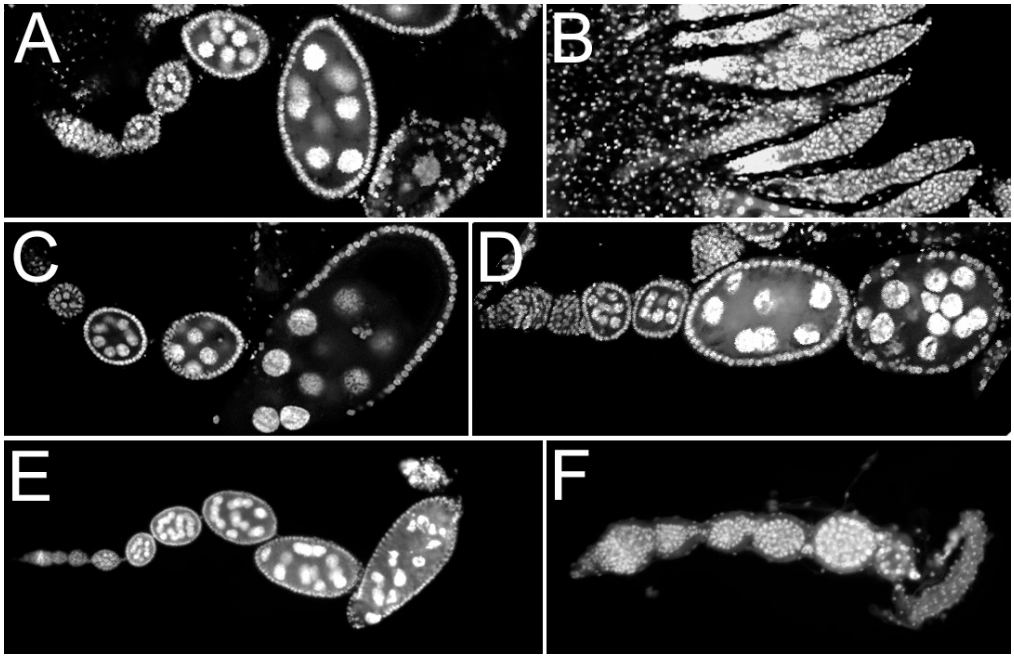


Figure 2.4. Grk protein accumulates in nurse cells of S^1 $aret^{PD}/aret^{PA}$ ovaries.

Stage 7 egg chambers are shown for each genotype, with nuclei in red (ToPro-3) and Grk in green. In wild type egg chambers Grk protein is restricted to the oocyte (A). *aret* mutants ($aret^{PD}/Df(2L)esc\text{-}P2\text{-}0$) also have detectable Grk only in the oocyte (B). When S^1 is also present, in S^1 $aret^{PD}/aret^{PA}$, ectopic Grk can be detected in the nurse cell cytoplasm (C) as well as the oocyte (C', a different focal plane). Grk protein is also concentrated at nurse cell boundaries (arrows in D, a higher magnification view of a part of C). The *aret* mutant combination is stronger in B ($aret^{PD}/Df(2L)esc\text{-}P2\text{-}0$) than in C ($aret^{PD}/aret^{PA}$) to allow similarly staged egg chambers to be compared.

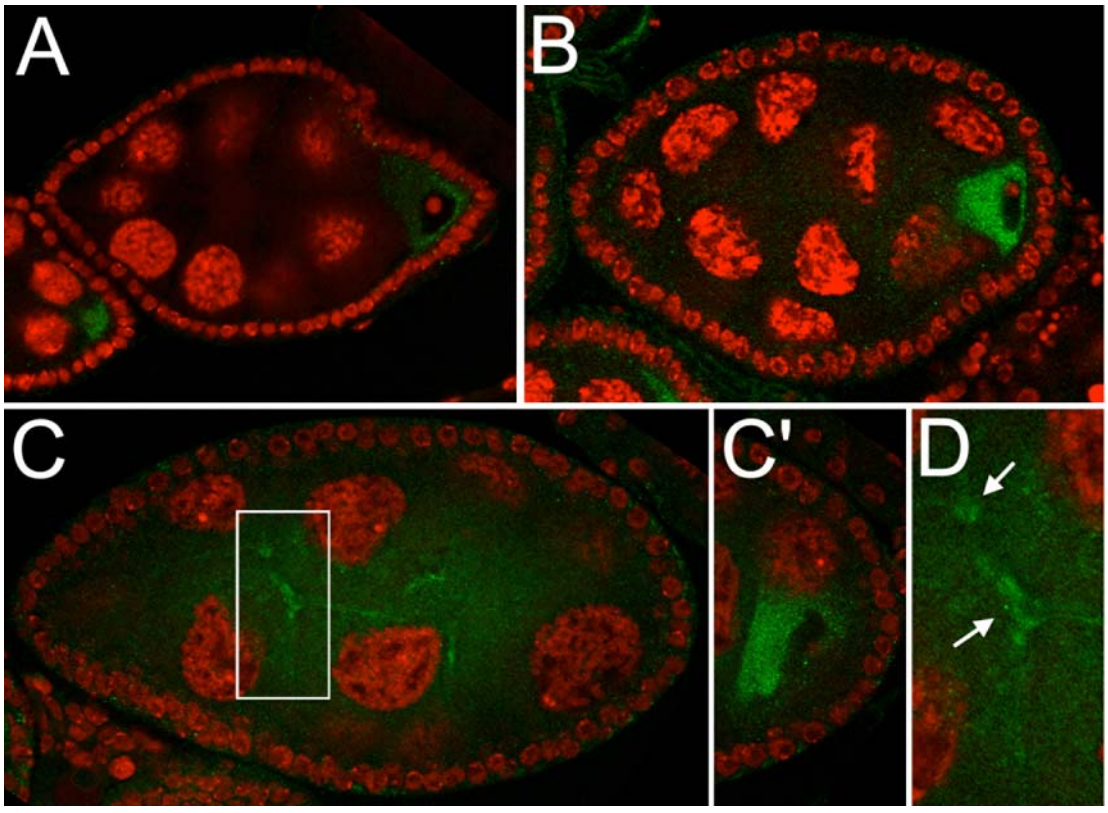


Figure 2.5. Mutation and overexpression of *Lk6* leads to opposite effects on the *aret* phenotype.

Ovaries from $aret^{PD}/aret^{QB}$ females arrest oogenesis at stage 6 or 7 (A), but the arrest is delayed when the females are also heterozygous for *Df(3R)M-Kx-1*/+ (B). A single copy of the EP(3)0886 P element insertion in *Lk6* has a similar effect (C). Conversely, overexpression of *Lk6* using EP(3)0886 and a germline GAL4 driver (matα4-GAL4-VP16#6) enhances the *aret* phenotype (D). The presence of the GAL4 driver alone does not change the *aret* phenotype (E). In all cases the *aret* combination was $aret^{PD}/aret^{QB}$, and the ovaries were stained with ToPro-3.

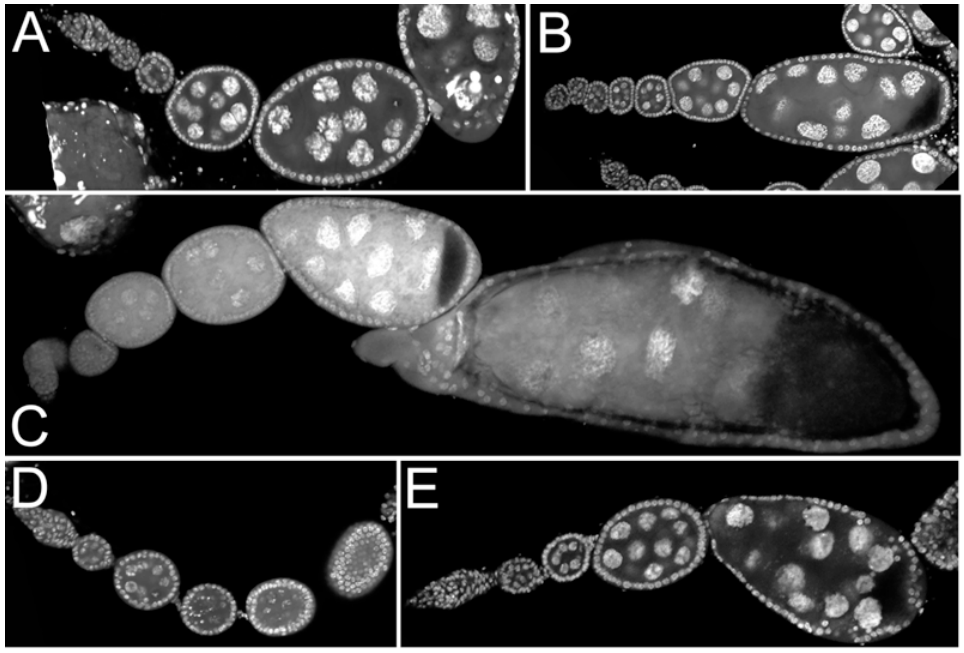


Figure 2.6. Genetic interaction of *Dl* with *aret*.

A. An arrested ovariole of an $aret^{QB}/aret^{QB}$ female. Some of the egg chambers show an overproliferation of the germ line (identified by Vas, red staining in all panels; an example is shown at higher magnification in A'). Green staining in all panels is Hts.

B. An ovariole of an $aret^{QB}/aret^{QB}$; Dl^{9P}/+ female. Only a single large egg chamber is present, and it has many germline cells.

C-E. Higher magnification view to show the distribution of Hts, a fusome marker, in wild type (C), $aret^{QB}/aret^{QB}$ (D) and $aret^{QB}/aret^{QB}$; Dl^{9P}/+ (E). The fusome is branched and extends between cells in wild type (arrowheads), but appears as dots in both mutants (arrows), indicating that they have undergone complete cytokinesis.

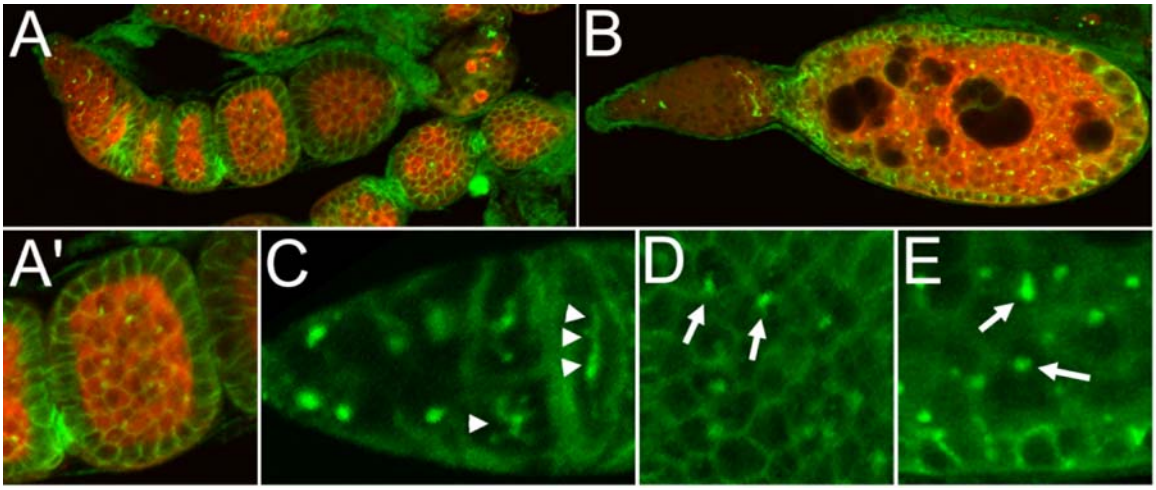


Figure 2.7. The combination of *aret*^{PD}/*aret*^{QB} and *Dl*^{9P}/+ mimics the *Dl*/*Dl* phenotype.

A-C. The different classes of *aret*^{PD}/*aret*^{QB}*; Dl*^{9P}/+ phenotypes. A shows a single large egg chamber that has formed by fusion or overproliferation. B and C show ovarioles with random or A/P incomplete egg chamber fusions, respectively. D and E are controls for comparison, *aret*^{PD}/*aret*^{QB} (D and D') and *aret*^{QB}/+*; Dl*^{9P}/+ (E and E'). The absence of stalk cells between incompletely fused egg chambers is shown in C', while stalk cells are visible in the controls (D' and E'). All panels show Hts staining, which is strongest in somatic cells and is especially enriched in the stalk cells.

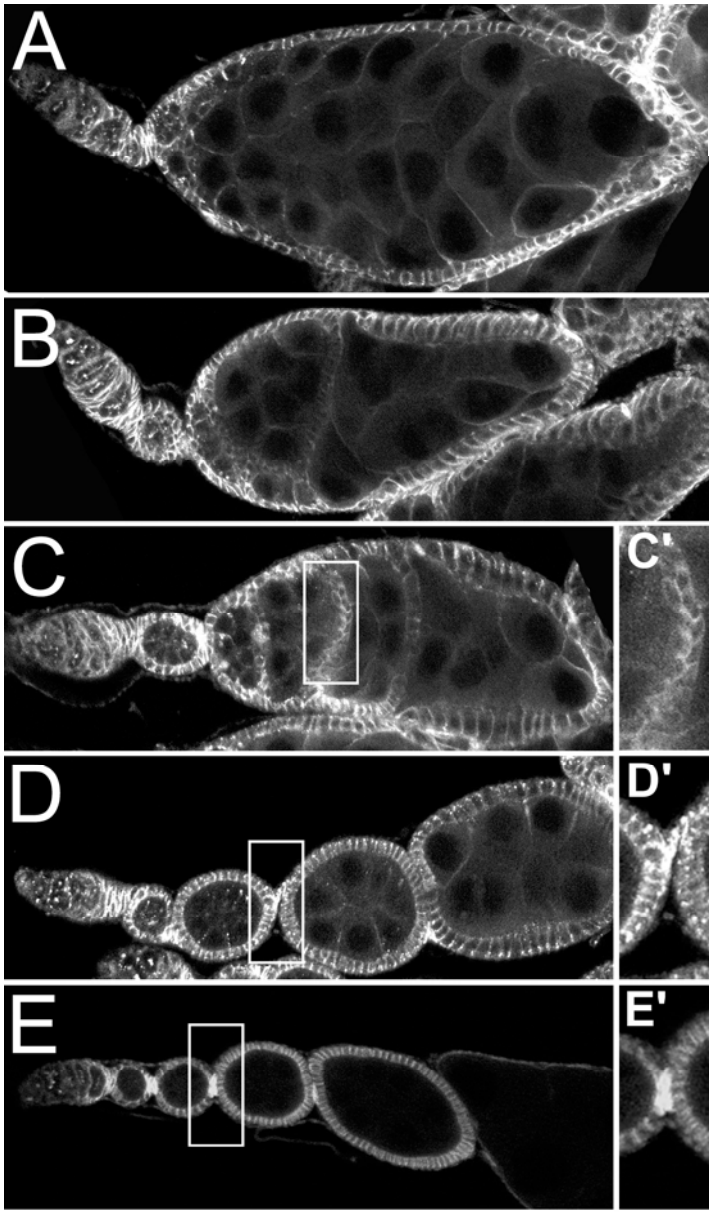
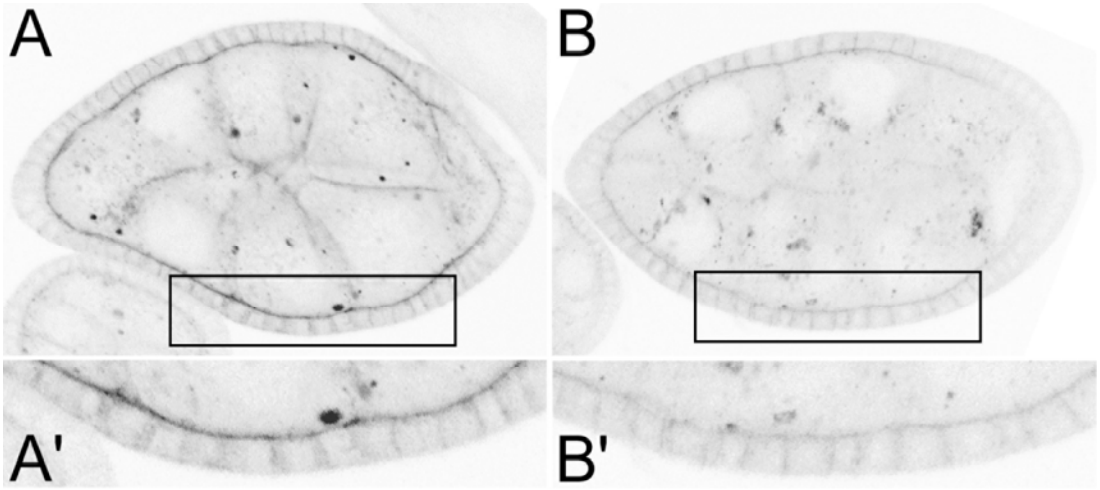

A
B
C
C'
D
D'
E
E'

Figure 2.8. *aret* mutant ovaries are deficient in Dl protein.

A. Dl in wild type (A) and $aret^{PD}/aret^{QB}$ (B) egg chambers. In each case the protein was detected by immunofluorescence using a confocal microscope with the same laser power and gain settings. In the wild type egg chambers, during stages 5-7, Dl is enriched at the surface of germline cells, especially where they appose follicle cells (A'), and dispersed throughout the cytoplasm. The overall distribution is similar in the *aret* mutant, but the amount of Dl protein in or closely associated with the membranes is significantly reduced (B').



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Chapter 3: Bruno both represses and activates translation by binding to different BREs in the *oskar* mRNA 3' UTR

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ABSTRACT

Deployment of Oskar protein at the posterior pole of the Drosophila oocyte relies on translational regulation. Bruno binds to BREs in the *oskar* mRNA and represses its translation, by a mechanism proposed to entail recruitment of Cup and thereby inactivation of eIF4E. Unexpectedly, Bruno mutants lacking a protein interaction domain and unable to bind Cup (and other proteins) still efficiently block accumulation of Osk. These mutants are indeed deficient in repression, but also prevent activation of *oskar* mRNA translation. Selective mutation of the two clusters of BREs in the *oskar* 3' UTR reveals opposing roles: BREs near the coding region act in repression, while BREs near the poly(A) tail mediate activation. Bruno is both a repressor and activator of translation.

INTRODUCTION

Formation of the body plan of the Drosophila embryo relies on the action of several localized determinants [reviewed in (Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001)]. Bicoid (Bcd) protein, which is concentrated at the anterior of the embryo, specifies anterior fate. Two determinants - Oskar (Osk) and Nanos (Nos) act sequentially in posterior patterning. Osk protein is localized to the posterior pole of the oocyte and initiates formation of the pole plasm, which contains the activities for both posterior body patterning and germ cell formation. Nos protein is synthesized exclusively in or near the pole plasm, and serves as the localized component of the posterior patterning activity. Gurken (Grk) protein is highly concentrated at an anterodorsal position in the oocyte, and establishes the dorsoventral body axis. Each of these determinants has a potent patterning activity, such that overexpression or mislocalization can dramatically alter body patterning. For example, increasing the dosage of the *osk* gene progressively posteriorizes the embryo. Mild overexpression causes weak anterior defects, while higher levels lead to replacement of all head and thoracic segments with a mirror image duplication of posterior abdominal segments, the bicaudal phenotype (Smith et al., 1992). Similarly, specific misexpression of Oskar at the anterior efficiently produces bicaudal embryos (Ephrussi and Lehmann, 1992). Thus, proper deployment of the determinants is a critical step in embryonic pattern formation.

Several mechanisms are used to ensure that the localized determinants appear only at the correct positions and in the desired amounts. The *bcd*, *osk*, *nos* and *grk* mRNAs are all prelocalized to the appropriate region within the oocyte or embryo (St Johnston, 1995). Each mRNA is also translationally regulated, such that protein distribution is dictated by the pattern of mRNA localization (Lipshitz and Smibert, 2000).

This translational control can be complex. In the case of *osk* mRNA, there is evidence for at least two forms of translational repression, as well as localization-dependent activation. Co-translational repression is achieved after initiation of translation, and prevents the accumulation of Osk protein from *osk* mRNA that is associated with polysomes (Braat et al., 2004). This phenomenon is reminiscent of microRNA (miRNA) mediated translational control (Bartel, 2004), and certain mutants with defects in the related process of RNA silencing accumulate Osk protein precociously (Cook et al., 2004; Tomari et al., 2004). Thus co-translational repression may involve miRNAs.

A second form of repression is controlled by sequences in the *osk* mRNA called BREs, which are binding sites for Bruno (Bru) . The BREs are clustered in two regions, AB and C, at opposite ends of the *osk* 3'UTR: the AB region is near the coding region, while the C region is close to the polyadenylation site. Mutation of all of the BREs largely eliminates Bru binding in vitro and leads to precocious Osk protein expression in vivo, implicating Bru as a translational repressor (Kim-Ha et al., 1995; Webster et al., 1997). A substantial body of evidence supports this conclusion, including the demonstration that Bru and BREs are required for repression in vitro (Castagnetti et al., 2000; Lie and Macdonald, 1999a).

The mechanism by which Bru represses *osk* mRNA translation appears to involve Cup. Mutants of *cup* display precocious accumulation of Osk, and Cup physically interacts with Bru. Cup, like a number of translational control factors, binds to translation initiation factor eIF4E. In doing so it prevents eIF4E from binding to eIF4G, an interaction that is required for conventional cap-dependent initiation of translation. Thus, Bru has been proposed to repress *osk* mRNA translation by recruiting Cup, which in turn binds eIF4E and blocks initiation (Nakamura et al., 2004).

Here we show that mutants of Bru unable to bind Cup (and other interactors) effectively block accumulation of Osk. Superficially, these results appear to be inconsistent with the model for Bru-mediated repression. However, complementary analyses of the roles of the different clusters of BREs in the *osk* mRNA and of the properties of interaction-defective Bru proteins reveal that Bru has two activities: the recognized role in repression, which may well require binding to Cup, and a novel role in activation of translation. Loss of both activities accounts for the phenotype of the interaction-defective Bru proteins.

RESULTS

Identification of a domain required for Bru dimerization

Bru binds to several proteins, including Vasa (Vas)(Webster et al., 1997), Apontic (Apt) (Lie and Macdonald, 1999b), Cup (Nakamura et al., 2004) and itself (data presented here). Three of these interactions -- Bru/Apt, Bru/Bru (data presented here), and Bru/Cup -- can be monitored by the yeast two hybrid assay, providing a simple means of mapping the binding domain or domains of Bru. The Bru/Bru combination provides the most robust interaction, and was used for initial mapping experiments. Three groups of systematic deletion mutants were tested. In one group the RRM RNA binding motifs were removed, either individually or in combination. The two other groups of mutants consisted of progressively larger deletions from either the amino or carboxyl terminus of the protein. The results indicated that sequences between RRMs 2 and 3 (amino acids 318-513; referred to as the interaction domain) are crucial for binding (data not shown). To confirm this conclusion, mutant proteins lacking part of the interaction domain were tested, as were proteins consisting of only the interaction domain (Fig. 3.1A). When the first half of the interaction domain is deleted, the Bru/Bru interaction is severely disrupted, even though the level of the mutant protein is not dramatically reduced. The interaction domain alone binds to Bru and to itself, although the strength of the interaction is reduced. When the interaction domain is divided into two parts, the first half retains partial binding activity to full length Bru, but not to itself, and the second half does not bind Bru (Fig. 3.1B). These results strongly indicate that the interaction domain mediates Bru dimerization.

In an attempt to more narrowly define the important elements of the interaction domain, a library of randomly mutagenized plasmids expressing the domain were screened for binding to Bru. Two mutants defective in binding were identified from screening ~3000 transformants. One has a his to arg change at position 391 (H391R), and the other has two changes, gln 393 to his and gly 400 to glu (Q393H G400E; the mutant originally carried two additional mutations at positions 430 and 470, but removal of these mutations did not restore the interaction). To estimate the fraction of amino acids in the interaction domain tested by this approach, plasmids from 15 transformants that retained Bru binding in the assay were sequenced. They had from 1 to 9 amino acid changes in the interaction domain each, with a median of 2 changes. Thus about 2 x 3000 amino acid change mutations were sampled in the screen, and mutants with changes at most of the amino acids in the 195 amino acid interaction domain should have been tested. The low frequency at which binding mutants were recovered suggests that the interaction is relatively insensitive to single amino acid changes, and may thus involve a broad surface of the protein. Although the H391R and Q393H G400E mutants very strongly disrupt the interaction of the isolated interaction domain with Bru, when placed in the context of Bru they have little or no effect. Because the interaction domain/Bru binding is weaker than Bru/Bru binding, it appears to be more sensitive to changes.

Two additional mutants that affect the Bru/Bru interaction were constructed (Fig. 3.1A). An 8 amino acid deletion near the point mutants described above, BruΔ382-389, has reduced binding, as does Bru::GFP, a protein created by fusion of GFP to the carboxyl terminus of full length Bru; presumably, fusion with GFP alters the conformation of Bru or limits accessibility to a binding surface, and thus impairs dimerization.

Bru dimerization mutants are also defective in Cup binding

Bru mutants used to identify the domain required for dimerization were also tested for interaction with Apt and Cup (Fig. 3.1C). The interactions of Bru with itself and with Cup are similar, in that the BruΔ334-416 mutant fails to bind to either protein, and the binding to Bru and Cup are reduced in proportionate degrees for BruΔ382-389 and Bru::GFP proteins. Furthermore, the isolated Bru interaction domain binds to both Bru and Cup. The only substantial difference is that the complete and partial interaction domains both bind to Bru, while only the complete domain binds to Cup. We confirmed that BruΔ334-416 fails to bind either Bru or Cup using pull-down assays. GST::Cup and GST::Bru both bind Bru, but neither binds BruΔ334-416 (Fig. 3.1D,E).

The large and small deletions of the Bru interaction domain prevent or reduce binding to Apt, respectively. However, in contrast to the results with Bru and Cup, the isolated Bru interaction domain fails to bind Apt, and Bru::GFP retains strong Apt binding (Fig. 3.1C). Apt could bind to Bru via the interaction domain, with the varying sensitivities to the different mutants reflecting the use of a different part of the domain. However, because Apt does not bind to the isolated domain, we cannot exclude the possibility that Apt binds to a different part of Bru whose folding is indirectly altered as a consequence of a deletion within the interaction domain.

Testing the role of the Bru interaction domain in vivo

The Bru/Cup interaction has been proposed to mediate translational repression (Nakamura et al., 2004). By this model the mutants defective in Cup binding - BruΔ334-416, BruΔ382-389 and Bru::GFP - are predicted to lack repressive activity. To investigate the role of the interaction domain in vivo, and to test this prediction, we relied on an overexpression assay. GAL4-driven expression (Brand and Perrimon, 1993) of a wild type *bru* cDNA under UAS control enhances repressive activity. Females expressing the

bru cDNA produce a large fraction of embryos lacking some or all abdominal segments, and Osk protein accumulation is reduced or undetectable (Snee et al., submitted; Figs. 3.2 and 3). In this assay the transgenic Bru can compete with the endogenous protein for binding to BREs, allowing a mutant form of Bru to interfere with a Bru-dependent process. An alternate rescue assay, in which transgenic Bru is expressed in a *aret* mutant [the *aret* gene encodes Bru (Webster et al., 1997)], is not suitable. Although transgenic Bru does rescue, the rescue is not complete (Filardo and Ephrussi, 2003)(Snee et al., submitted). More importantly, *aret* mutants display no obvious defect in regulation of *osk* (Schüpbach and Wieschaus, 1991; Webster et al., 1997), and thus the rescue assay cannot be used to study the role of Bru in that process.

Surprisingly, Bru mutant proteins impaired in Cup binding do not have the predicted phenotype. Each of the mutants is more effective than wild type Bru in producing embryos lacking abdominal segments (Fig. 3.2). Expression of wild type *P[UAS-bru]* in the ovary results in about 70% of embryos lacking all abdominal segments. For the interaction-defective mutants, ~80-95% of their embryos lack all abdominal segments. This enhanced inhibition of posterior patterning is accompanied by a more severe disruption of Osk accumulation: the fraction of oocytes with reduced or undetectable levels of Osk is dramatically increased (Fig. 3.3). These results appear to suggest that the interaction of Bru with Cup may not be required for translational repression of *osk* mRNA. An alternate interpretation, which accommodates the model that the Bru/Cup interaction mediates repression, is that Bru has an additional role in activation of *osk* translation. In this scenario the mutant Bru protein dominantly interferes with activation, and thereby obscures defects in repression. Two lines of evidence, one focusing on the Bru binding sites in *osk* mRNA, and one selectively assaying Bru repressive activity, support the latter interpretation.

The different BREs of *osk* mRNA have different roles

Bru binds to multiple sites, BREs, in the *osk* mRNA 3' UTR. Some of the BREs are clustered in the AB region near the beginning of the 3' UTR, and others are in the C region close to the poly(A) tail. The original demonstration that Bru and the BREs act in repression of *osk* translation made use of an *osk* genomic DNA transgene (which in the wild type form rescues osk^- mutants) in which all of the BREs had been inactivated by point mutations. Osk protein was expressed ectopically from this transgene, leading to excessive posterior patterning activity and the formation of posteriorized embryos with anterior defects or complete replacement of the anterior by a duplicated posterior (Kim-Ha et al., 1995).

We reevaluated the roles of the different BREs by testing similar *osk* genomic DNA transgenes in which the BREs of only a single region were mutated, either AB (*P[oskAB$^-$]*) or C (*P[oskC$^-$]*). Transgenes were introduced into osk^+ females, and their embryos were examined for patterning defects. Two independent lines of each transgene were tested; there was some phenotypic variation between the different lines, but these differences were minor in comparison to the strikingly different consequences of mutating the different sets of BREs.

Osk protein is limiting for posterior body patterning, and mothers with extra copies of the osk^+ gene produce posteriorized embryos [(Smith et al., 1992); Fig. 3.4]. This activity is enhanced for the *P[oskABC$^-$]* transgene, in keeping with previous results (Kim-Ha et al., 1995). The *P[oskAB$^-$]* transgene causes even more pronounced posteriorization of the embryos. This elevated posterior patterning activity is consistent with a role for the AB BREs in translational repression. In contrast, the *P[oskC$^-$]* transgene has dramatically reduced patterning activity, much less than that of the *P[osk$^+$]* transgene: the vast majority of embryos from mothers with the *P[oskC$^-$]*

transgene are wild type. The very limited activity of the *P[oskC^{-}]* transgene is not a consequence of reduced transcription or mRNA stability, as the level of *osk* transcripts is similar in ovaries of flies expressing each of the transgenes (Fig. 3.5B). In addition, mutation of the BREs has no effect on *osk* mRNA localization (Kim-Ha et al., 1995).

We extended analysis of the transgenes, examining levels of Osk protein in Osk protein-null [*osk^{54}/Df(3R)pXT103*] host flies and their embryos (Fig. 3.5). In ovaries the level of Osk protein from the *P[oskAB^{-}]* transgene is clearly greater than from the *P[osk^{+}]* transgene. The *P[oskABC^{-}]* transgene produces Osk at a level similar to that of the *osk^{+}* transgene [there is precocious accumulation of Osk (Kim-Ha et al., 1995), but this increase is minor when considered in the context of all of oogenesis, as in a western blot of ovaries], while the *P[oskC^{-}]* transgene produces less Osk than any of the other transgenes. In early embryos (0-2 hours after egg laying) the elevation of Osk conferred by mutation of the AB region BREs is no longer apparent, while the reduction in Osk expression due to mutation of the C region BREs is more pronounced. The temporal complexities in Osk expression revealed in our analysis merit further attention. Nevertheless, the clear and important conclusion for this study is that the AB and C region BREs have very different roles, acting to repress and to activate *osk* mRNA translation, respectively.

The Bru interaction domain is required for both translational repression and activation

Our evidence that different BREs have negative or positive roles in *osk* mRNA translation is consistent with the notion that Bru acts in both repression and activation of translation. By this model the interaction-defective Bru mutants bind *osk* mRNA at the C region BREs but fail to recruit an activating factor, and thus dominantly block activation of *osk* translation. The interaction-defective Bru mutants could also be defective in

repression, as predicted by the model for the mechanism of Bru repression in which Bru recruits Cup. However, in the absence of the activating step the loss of repression might not be detected. Evidence that the interaction-defective Bru mutants are defective in repression would further support the assignment of an activating function to Bru.

To test the interaction-defective BruΔ334-416 mutant for repressive activity we developed an assay that selectively monitors BRE-dependent repression of translation. Expression of the *osk* coding region under UAS/GAL4 control and without the *osk* 3' UTR, in transgene *P[UAS-osk]*, is translationally unregulated and the resulting embryos are bicaudal as a consequence of the ectopic *osk* activity [(Vanzo and Ephrussi, 2002) and Fig. 3.6]. Insertion of the *osk* AB region after the coding region of the transgene, to make *P[UAS-osk-AB]*, confers translational repression: the bicaudal phenotype is eliminated and all embryos have either wild type or have minor patterning defects (including loss of anterior structures, which is indicative of a low level of ectopic *osk* activity). Mutation of the BREs, in *P[UAS-osk-AB$^-$]*, greatly reduces repression (Fig. 3.6). Thus the *P[UAS-osk-AB]* transgene can be used to monitor the repressive activity of Bru independent of its presumed role in activation via the C region BREs. We can further increase the specificity of the assay by eliminating endogenous *osk* (in an *osk$^-$* background), such that all *osk*-dependent effects on embryonic body patterning must reflect the activity of the *P[UAS-osk-AB]* transgene.

If BruΔ334-416 is defective in translational repression, then by competing with endogenous wild type Bru for binding to BREs it should interfere with repression of *P[UAS-osk-AB]* translation. The consequence would be increased ectopic Osk expression and enhanced anterior patterning defects. Expression of *P[UAS-osk-AB]* in *osk$^-$* females produces embryos that lack all abdominal segments, since the *P[UAS-osk-AB]* transcripts are repressed and cannot rescue the *osk* mutant phenotype. A fraction of the embryos

have head defects, consistent with a low level of Osk accumulation due to incomplete repression (Webster et al., 1997). Co-expression of *UAS-bru* does not substantially alter the embryonic phenotype. However, co-expression of *UAS-bruΔ334-416* significantly increases the proportion of embryos with anterior defects (Fig. 3.7). We conclude that the interaction-defective form of Bru interferes with repression, consistent with the model in which the Cup/Bru interaction underlies repression. Thus the ability of the mutant Bru protein to block accumulation of Osk translated from the endogenous *osk* mRNA is not because it is functioning as a repressor; instead, the mutant Bru protein presumably competes with endogenous Bru for binding to BREs (including those in the C region), fails to recruit or bind an activator, and thus interferes with Bru-dependent translational activation.

BruΔ334-416 binds BREs, and this activity is required to block Osk accumulation

Our interpretation of the ability of BruΔ334-416 to interfere with regulation of *osk* mRNA translation, both negative and positive, invokes a competition with endogenous Bru for binding to BREs. To confirm that BruΔ334-416 retains BRE binding activity, UV-crosslinking assays were performed with ovarian extracts from females expressing the mutant protein. Both endogenous Bru and the transgenic BruΔ334-416 bind to BRE RNA (Fig. 3.8). This binding activity is essential for the activity of BruΔ334-416: a BruΔ334-416 transgene bearing point mutations that largely abolish Bru RNA binding activity (D.H, J. Jen, M. Snee and P.M.M., unpublished) has no substantial effect on embryonic body patterning (data not shown).

DISCUSSION

In the initial analysis of translational regulation of *osk* mRNA the BREs were defined as control elements that mediate repression. That conclusion was based on the phenotypic consequences of mutating all BREs: precocious expression of Osk, and body patterning defects (Kim-Ha et al., 1995). The subsequent development of in vitro translation systems that support Bru- and BRE-dependent translational repression reinforced the conclusion that Bru acts as a repressor when it binds to BREs (Castagnetti et al., 2000; Lie and Macdonald, 1999a). By selectively mutating the BREs from different parts of the *osk* 3' UTR we have now revealed a more complex picture of the role of the BREs, which act in both repression and activation of *osk* mRNA translation. The bifunctional nature of the BREs raises the question of whether Bru also mediates activation, and evidence presented here strongly argues for such an additional role. Specifically, we find that a mutant of Bru defective in at least two of its protein/protein interactions – with itself and with Cup - can block accumulation of Osk, yet the mutant is defective in repression. We infer that the mutant is also impaired for the activation function mediated by the C region BREs. This discovery explains the enigmatic observation that reduction of the amount of Bru (in an *aret* heterozygote) can partially suppress the Osk overexpression phenotype of a *Bic-C* mutant (Castagnetti and Ephrussi, 2003).

Repression by Bru has been proposed to occur through interaction with Cup, which then locally inactivates eIF4E (Nakamura et al., 2001). Our results are consistent with this model, as disruption of the Bru/Cup interaction interferes with repression.

The mechanism of translational activation by the *osk* C region BREs is unknown. Activation of other mRNAs often involves extension of the poly(A) tail, in a process

dependent on a cytoplasmic polyadenylation element (CPE) positioned close to the poly(A) tail, and a protein, CPEB, that binds to the CPE (Mendez and Richter, 2001). There is a CPE-like element (U_8GU) almost immediately adjacent to the *osk* C region BREs, and the Drosophila CPEB protein, Orb, has a positive role in accumulation of Osk (Castagnetti and Ephrussi, 2003; Chang and Peter, 2002). Moreover, Orb has been reported to coimmunoprecipitate with Bru (Castagnetti and Ephrussi, 2003). Despite these indications, we have been unable to detect any alteration in poly(A) tail length when accumulation of Osk is very efficiently blocked by expression of the BruΔ334-416 protein (data not shown).

Although the mechanism of Bru-dependent activation is unknown, several features of activation, and of the relationship between repression and activation, are noteworthy. First, the *P[oskC$^-$]* transgene is not completely inactive, and produces some Osk. This could indicate that the C region- and Bru-dependent activation serves to enhance translation, but is not essential. This interpretation is not readily reconciled with the ability of the interaction defective forms of Bru to efficiently block accumulation of Osk. An explanation is suggested by a reexamination of Bru binding to the *osk* C region. The C region, as defined originally and used for in vitro RNA binding assays, corresponds to a 72 bp XbaI to Sau3A restriction fragment near the 3' end of the *osk* transcript. Introduction of the BRE$^-$ mutations largely abolished Bru binding to an RNA from that fragment, and the same mutations were introduced into the complete *osk* gene to make the *P[oskC$^-$]* transgene. We find that a larger version of the C region, extending to the polyadenylation site, is a better substrate for Bru binding. Given this result, there are probably additional Bru binding sites in the larger C region, and these are not mutated in the *P[oskC$^-$]* transgene. Consequently, the *P[oskC$^-$]* transgene may retain partial

activation function, which would allow a low level of Osk to accumulate as is observed. Thus, the C region-dependent activation may well be essential.

Knowing when Bru-dependent activation occurs could provide insight into the mechanism. Bru could act prior to localization of *osk* mRNA by making the mRNA competent for translation, an activity that would initially be masked by the contemporaneous repression that prevents accumulation of Osk. Bru could act coincident with *osk* mRNA localization, overriding repression and triggering the onset of Osk accumulation. Or Bru could act after *osk* mRNA localization, during the late stages of oogenesis when there is substantial accumulation of Osk protein and a mechanism may exist to enhance translation (Snee et al., submitted). At present we are unable to rule out any option. It might appear that Bru cannot simply override its own form of repression, as loss of both activating and repressing Bru activities would lead to elevated Osk: this is not observed. However, the BruΔ334-416 protein is more effective in dominantly interfering with activation than with repression, at least in the assays used, and we cannot predict the overall effect of inhibiting both processes to different extents. One suggestion about the timing of Bru activation comes from the pattern of Osk accumulation from the *P[oskC$^-$]* transgene. Mutating the C region BREs reduces the level of Osk, both when tested in ovaries and in early embryos. However, the effect is strongest in embryos, raising the possibility that the activation step is specifically involved in a late phase of Osk accumulation. Recent evidence demonstrates that much of Osk protein appears very late in oogenesis (Snee et al., submitted), and this phase of expression might be subject to stage-specific forms of control.

One intriguing question about the dual roles of Bru is why binding to one set of BREs results in repression, while binding to other BREs confers activation. There are many situations in which a single type of binding site can exert either positive or negative

effects on gene expression, depending on which proteins are bound; the family of basic helix-loop-helix transcription factors provides a general example. However, it is much less common for a single nucleic acid binding protein to have opposing effects dependent on which regulatory site it binds, and for the protein to play a more positive role in each process than simply preventing another factor from binding to the regulatory site.

We envisage three possible models for the two functions of Bru (Fig. 3.9). In the first, Bru can recruit the same spectrum of factors independent of its binding site, but the consequences of Bru binding depend on its position within the mRNA. For example, binding at any site might promote repression, while activation would only be possible when Bru binds and recruits an activator in the immediate vicinity of the poly(A) tail. In the second model the different activities depend on other factors that can bind near Bru. For example, the *osk* C region might contain a binding site for a factor that cooperates with Bru to mediate activation. By the current model for Bru- and Cup-dependent repression, no protein binding near Bru in the *osk* AB region would be necessary, although there is no reason to exclude such a possibility. Finally, a third model invokes qualitative differences in the Bru binding sites in the AB and C regions of the *osk* 3'UTR. In this model the nature of the RNA substrate would dictate how Bru acts. For example, when bound to the AB region Bru might assume a conformation that enhances interaction with a repressive binding partner, such as Cup. Adoption of a different conformation when bound to the C region might favor binding of Bru to an activator.

Although we cannot yet distinguish between these models (and features of more than one could contribute to the actual mechanism), there is one intriguing difference between the Bru binding sites of the AB and C regions. The BREs were initially defined as a consensus sequence of U(A/G)U(G/A)U(A/G)U, but this description is probably incomplete. For example, the D. virilis *osk* mRNA 3' UTR also binds Bru, but in the AB

region there is only a single copy of the consensus, as opposed to four in the D. melanogaster *osk* AB region (Webster et al., 1994). Notably, EDEN-BP, a protein whose RRM RNA binding motifs are very closely related to those of Bru (Paillard et al., 1998), binds to multimers of UAUG or UGUA (Bonnet-Corven et al., 2002), sequences embedded within the BREs. There are seven copies of these tetranucleotides in the *osk* AB region (and the same number for D. virilis), and all are disrupted by the mutations that abrogate Bru binding. Similarly, there are four copies of the tetranucleotides in the C region, as originally defined, with all affected by the BRE$^-$ mutations (the D. virilis C region also has 4 copies). Thus, it seems likely that the Bru recognition site contains one or more copies of the tetranucleotides. In the AB region most copies of the tetranucleotides are separated from one another by gaps. In contrast, in the C region the tetranucleotides are invariably overlapping. This general pattern holds for the *osk* AB and C regions of all Drosophila species that have been sequenced. These differences between the AB and C regions might influence how Bru binds, and either directly or indirectly affect its conformation and choice of binding partners.

MATERIALS AND METHODS

Flies and transgenes

w^{1118} flies were used as the wild-type. All mutant flies are as described at FlyBase (http://flybase.bio.indiana.edu/). *P[UAS-bru]* and *P[UAS-bru::GFP]* were described previously (Snee and Macdonald 2004). *P[UAS-bru*Δ*334-416]* and *P[UAS-bru*Δ*382-389]* were constructed by deleting the PflMI-NotI fragment or PstI-PstI fragment from full-length *bru* cDNA, respectively; ligation restores the correct reading frame. The modified *bru* cDNAs were substituted into the *P[UAS-bru]* transgene. The

nosGAL4VP16 (Van Doren et al., 1998) and maternal alpha tubulin (Martin and St Johnston, 2003) GAL4 drivers were used to express UAS transgenes.

The *P[osk^{+}]* and *P[oskABC^{-}]* transgenes were described previously (Kim-Ha et al., 1995). In the *P[oskABC^{-}]* transgene the mutant AB region has the sequence GAATTCGCTTAGTTTTAATTAGTTTTTAATTTTCCATTGTTCTCTGTCTTTGTTA TTTTAGATTTTCGTGCACTTGTCCTAGTCCATTATTTTAGATTATTTTGGGTTT TGGTTTCTTAGTTAGATTTAAA (an EcoRI-DraI fragment), and the mutant C region has the sequence TCTAGATAGCTATCTACTATTTATAAACTTATTTATTGTCTTGAATTTGAGTTA ACTTGAGTTATTGATGGTGATC (an XbaI-Sau3A fragment). The *P[oskAB^{-}]* and *P[oskC^{-}]* transgenes contain the same genomic DNA fragment as *P[osk^{+}]* and *P[oskABC^{-}]* and are wild type except for the mutations within the AB or C regions, which are as described above.

The *UAS-osk* series of transgenes contain the *osk* coding region as a BamHI fragment inserted into the BamHI site of the pUASp vector (Rorth, 1998). The *osk* coding region fragment is largely from an *osk* cDNA, except that a short genomic segment beginning at the BamHI site was added to the 5' end, and the 3' BamHI site was introduced just after the stop codon. For *P[UAS-osk-AB]* and *P[UAS-osk-AB^{-}]* the wild type or mutant AB region was first inserted into the BamHI site of pUASp as a BamHI-BglII fragment prior to addition of the osk coding region.

Transgenic flies were generated following standard procedures.

Yeast two hybrid assays and mutagenic PCR screen

The full-length *bru* cDNA was subcloned into yeast vector pGilda (Clonetech). Systematic deletion mutants were generated using PCR and subsequently cloned into yeast vector pJG4-5. The full-length *apontic* cDNA was subcloned into pJG4-5 as

described (Lie and Macdonald, 1999b). The *cup* cDNA in yeast vector pGAD10 was a gift from Robin Wharton (Verrotti and Wharton, 2000). Yeast stain EGY48 was transformed with reporter plasmid pRB1840 (Golemis and Brent, 1997) and appropriate two hybrid plasmids. β-Galactosidase activity was determined using a liquid assay (Burke et al., 2000).

pJG4-5-ID::GFP was constructed by subcloning a *GFP* cDNA at the 3' end of a *bru* cDNA fragment that encodes the interaction domain (amino acids 318-513). The resulting plasmid served as the DNA template for mutagenic PCR. The PCR mixture contained: 0.4 μg DNA template, 1 X Taq polymerase buffer (Invitrogen), 0.8 mM dNTPs (Promega), 18 pmol primers, 5 unit of Taq polymerase (Invitrogen) and 0.2 mM $MnCl_2$ in a 100 μl reaction. The 5' primer is 5'-TCCTACCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTGGCTCGAGCCAATCTCTGGAAC-3'. The 3' primer is 5'-AATTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATGGTACCCCGCAGCGGCGA-3'. PCR reactions were done with 60°C as annealing temperature and repeated 30 cycles. 40 μl of PCR-amplified fragments were co-transformed with 100 ng pJG4-5-ID::GFP (gapped with EcoRI and blunt-ended with Klenow polymerase) into yeast carrying pGilda-bru and reporter pRB1840. Transformants were grown on glucose plates selecting for the presence of all plasmids and subsequently replicated to galactose plates assaying for interaction. Colonies on galactose plates were scaned in a FluorImager SI (Molecular Dynamics) for the presence of GFP signal to eliminate PCR events which resulted in nonsense mutations or frame shifts. Colonies that tested positive for GFP signal and failed to show interaction with Bru were selected as positives. DNAs were subsequently extracted from these positive clones and insertions were analyzed by sequencing.

Protein expression in E. coli and GST pull-down assays

GST::Bru was constructed by subcloning full-length *bru* cDNA into pGEX-2T (Amersham). GST::Cup was a gift from Robin Wharton (Verrotti and Wharton, 2000). The Bru proteins used for binding were tagged at the amino terminus with six histidine residues, and were expressed using pET15b (Novagen) in E. coli CodonPlus RP/RIL (Stratagene). GST::Bru, GST::Cup or GST alone was first immobilized on glutathione sepharose (Amersham) and subsequently incubated with his-tagged Bru or BruΔ334-416 for 1 hour. The beads were spun down, washed with GST binding buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, protease inhibitors, 10% Glycerol) and boiled in SDS loading buffer. Elutes were separated by SDS PAGE and analyzed by western blotting. Rat anti-Bru antibody was used at 1:20,000 to detect Bru protein.

Detection of proteins and mRNAs

Immunostaining and *in situ* hybridization of ovaries were carried out as described (Kim-Ha et al., 1995). Rabbit anti-Osk antibodies were used at 1:2,000. Rat anti-Stau antibodies were used at 1:100. Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope.

Ovaries from ~20 females, fed on yeast for 2-3 days, were dissected in PBS and immediately transferred to 50 μl 1xSDS loading buffer. Ovaries were homogenized in 1xSDS loading buffer, boiled for 5 minutes and spun for 10 minutes at 13,000 rpm. The supernatant was used for western analysis. Early (0-2 hour) embryos were collected on yeasted apple juice plates, dechorionated in 50% bleach for 3 minutes, and immediately transferred to 50 μl 1xSDS loading buffer. Embryos were subsequently treated the same as ovaries for western analysis.

Ovaries from ~10 females, fed on yeast for 2-3 days, were dissected in PBS and immediately transferred to 250 μl Native Buffer (50 mM Tris [pH7.4], 0.25 M KCl, 25

mM $MgCl_2$, 50 units/ml RNase inhibitor 1 μg/ml pepstatin, 1 μg/ml leupeptin, 5 mM benzamide, 0.5 μM Pefabloc) on ice.The ovaries were washed, resuspended in 250 μl Native Buffer and homogenized. 750 μl Tri-Regent LS (Molecular Research Center, Inc) was added and RNA was extracted according to the manufacturers protocol. Finally, RNA was resuspended in 20 μl water (DEPC treated) and 1 μl was used for the RNase protection assay (Ambion, Inc).

UV cross-linking

RNA binding assays were performed as described (Kim-Ha et al., 1995) with ovary extract and probes from the *osk* AB (EcoRI to DraI) and C (XbaI to XbaI) regions.

Phenotypic analysis

Embryos were collected from young females in small population cages, aged for at least 24 hours, and cuticles prepared and mounted for examination.

ACKNOWLEDGEMENTS

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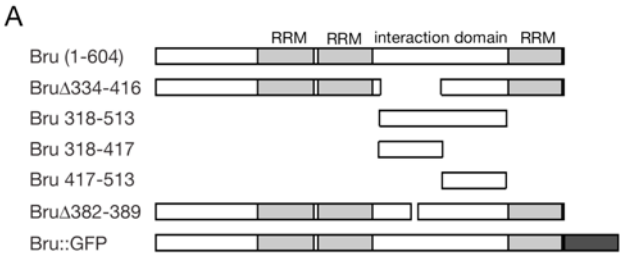
FIGURES

Figure 3.1. A domain of Bru mediates dimerization and interaction with Cup.

(A) Schematic diagram of wild type and mutant Bru proteins. The three RRM (Burd and Dreyfuss, 1994) RNA binding domains are lightly shaded, and GFP (not to scale) is dark grey. (B-C) Interactions measured by yeast two hybrid assays, with panel B showing interactions between various forms of Bru and panel C showing interaction of various forms of Bru with full length Bru, Apt and Cup. The strength of the interaction, as estimated by level of β-galactosidase activity, is denoted by the number of +'s where each + corresponds to 5 units of activity (Burke et al., 2000).
(D-E) BruΔ334-416 fails to interact with GST::Cup (D) and GST::Bru (E) in protein pull-down assays. GST fusion protein or GST alone attached to glutathione-sepharose beads was incubated with Bru or BruΔ334-416 proteins from E. coli. The amount of bound protein was detected by Western blotting with anti-Bru antibody. The positions at which Bru and BruΔ334-416 (labeled BruΔ) migrate are indicated at left. Lanes 1 and 2 in both panels represent 20% of the amount of input protein in the other lanes. In panel E the GST::Bru protein is partially degraded, as evident from lane 5. The stronger bands of degraded protein in lanes 3-5 are indicated with dots. Note that the strong band of input Bru in lane 3 migrates apart from the GST::Bru degradation products, and the position at which BruΔ334-416 migrates is distinct from the lower GST::Bru degradation product.

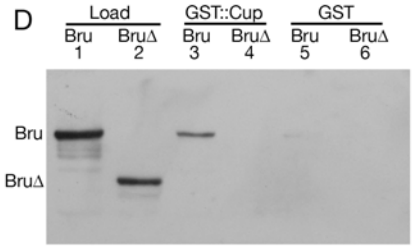


B

	Interaction
Bru / Bru	++++++
BruΔ334-416 / Bru	-
Bru 318-513 / Bru	++++
Bru 318-513 / Bru 318-513	++
Bru 318-417 / Bru	++++
Bru 318-417 / Bru 318-417	-
Bru 417-513 / Bru	-

C

	Bru	Apt	Cup
Bru	++++++	++	++
BruΔ334-416	-	-	-
Bru 318-513	++++	-	+++
Bru 318-417	++++	-	-
Bru 417-513	-	-	-
Bru::GFP	+	++	-
BruΔ382-389	+++	+	+



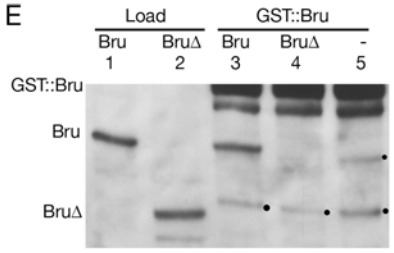
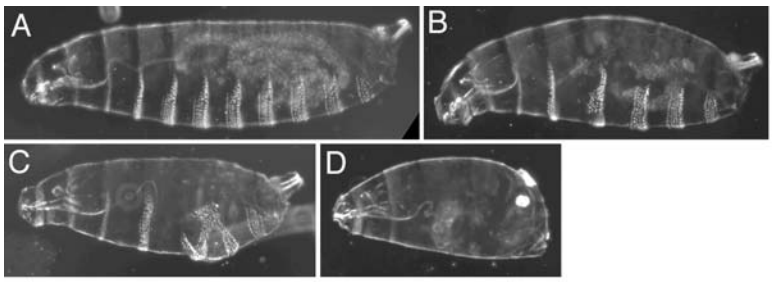


Figure 3.2. The Bru interaction domain is not required for efficient inhibition of posterior body patterning activity.

Examples of the different classes of cuticular phenotypes are shown in A-D, ranging from wild type (A) to partial (B and C) or complete (D) loss of abdominal segments. The distribution of embryos in the different classes is presented in E for two independent transgenic lines each of *P[UAS-bru]* and the three interaction-defective *bru* mutants. For all genotypes one copy of the UAS transgene was present, and the GAL4 driver was a single copy of *nosGAL4VP16*. The *nosGAL4VP16* driver by itself causes a small fraction of embryos to have minor anterior defects; these are not noted in E.

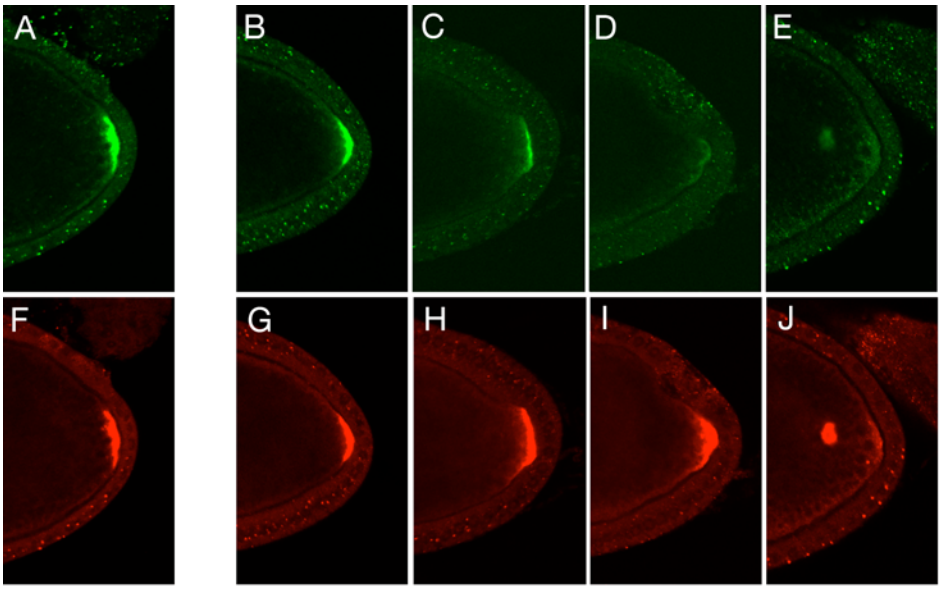


E

Transgene	wild type (%)	1-7 abdominal denticle belts (%)	0 abdominal denticle belts (%)	n
none	100	0	0	300
P[UAS-bru]-1	10	16	74	163
-2	24	10	66	232
P[UAS-bruΔ334-416]-1	15	2	83	463
-2	14	2	84	463
P[UAS-bruΔ382-389]-1	7	1	92	320
-2	10	1	89	331
P[UAS-bru::GFP]-1	4	2	94	120
-2	1	1	98	552

Figure 3.3. Interaction-defective forms of Bru display enhanced ability to block accumulation of Osk protein.

Osk (A-E) and Stau (F-J), which also serves as a marker for the distribution of *osk* mRNA, were simultaneously detected in stage 10 oocytes. The oocyte in A and F is wild type, while the remaining four oocytes are expressing forms of Bru under UAS/GAL4 control (the *nosGAL4VP16* driver was used). The examples in B-E correspond to the categories of Osk protein level and distribution indicated in the summary in panel K: normal Osk in B, reduced Osk in C, little or no detectable Osk in D, and Osk in a 'floating body' in E. The persistent posterior concentration of Stau, even when Osk levels are greatly reduced , indicates that *osk* mRNA localization is relatively normal and thus not affected by the Bru mutant proteins. The floating bodies are very rare, and only seen in significant numbers when *P[UAS-bru::GFP]* is expressed. Two observations suggest that they form by detachment from the posterior cortex. First, in some examples they remain linked to the posterior. Second, they are most frequently found near the posterior.

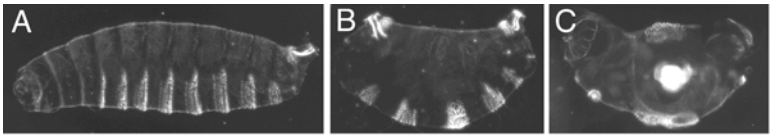


K

Transgene	normal Osk (%)	reduced Osk (%)	litte or no Osk (%)	floating body (%)	n
P[UAS-bru]	54	26	20	0	90
P[UAS-bruΔ334-416]	4	16	77	3	137
P[UAS-bru::GFP]	5	2	81	12	42

Figure 3.4. BREs in the *osk* mRNA AB and C regions have different roles.

(A-C) Examples of different classes of cuticular phenotypes from embryos of mothers expressing wild type or BRE$^-$ versions of transgenic *osk*, in addition to expression of the endogenous *osk*$^+$ gene. A, anterior defects; B, bicaudal; and C, severe bicaudal. (D) Distribution of embryos in the different phenotypic classes. Data are presented for two independent transgenic lines for each transgene. In all cases the females were homozygous for the transgene-bearing chromosome, and thus carried two copies of the transgene.



D

Transgene	wild type (%)	anterior defect (%)	bicaudal (%)	severe bicaudal (%)	n
P[osk$^+$]-1	2	29	58	11	358
-2	12	52	36	0	356
P[oskABC$^-$]-1	0	0	65	35	167
-2	0	2	78	20	232
P[oskAB$^-$]-1	6	0	31	63	17
-2	0	0	0	100	44
P[oskC$^-$]-1	95	4	1	0	175
-2	83	17	0	0	58

Figure 3.5. Mutation of *osk* AB and C BREs have opposing effects on Osk accumulation.

(A) Protein accumulation from wild type and BRE$^-$ *osk* transgenes, monitored by western blot analysis in ovaries and embryos. All transgenes were tested in the protein null *osk*^{54}*/Df(3R)pXT103* background, and so the Osk signal is due entirely to expression from the transgene. Tubulin levels were monitored in the same samples to control for differences in the amount of protein among different genotypes. In ovaries the *oskAB*$^-$ transgene directs accumulation of the most Osk, while the lowest levels are observed for the *oskC*$^-$ transgene. The deficit in Osk accumulation from the *oskC*$^-$ transgene is even more pronounced in early (0-2 hour) embryos, while the *oskAB*$^-$ transgene now behaves more like *osk*$^+$.

(B) Levels of *osk* mRNAs in transgenic flies. RNase protection assays were performed on ovarian RNA from flies expressing no transgene (*w*^{1118}) or a single copy of the indicated transgene. The assay detects both endogenous and transgenic *osk* transcripts, but does not distinguish between them. Levels of *rp49* mRNA were assayed in the same samples to control for differences in amount of RNA in the different genotypes. The substantial differences in Osk protein accumulation from different transgenes seen in panel A cannot be attributed to differences in mRNA levels.

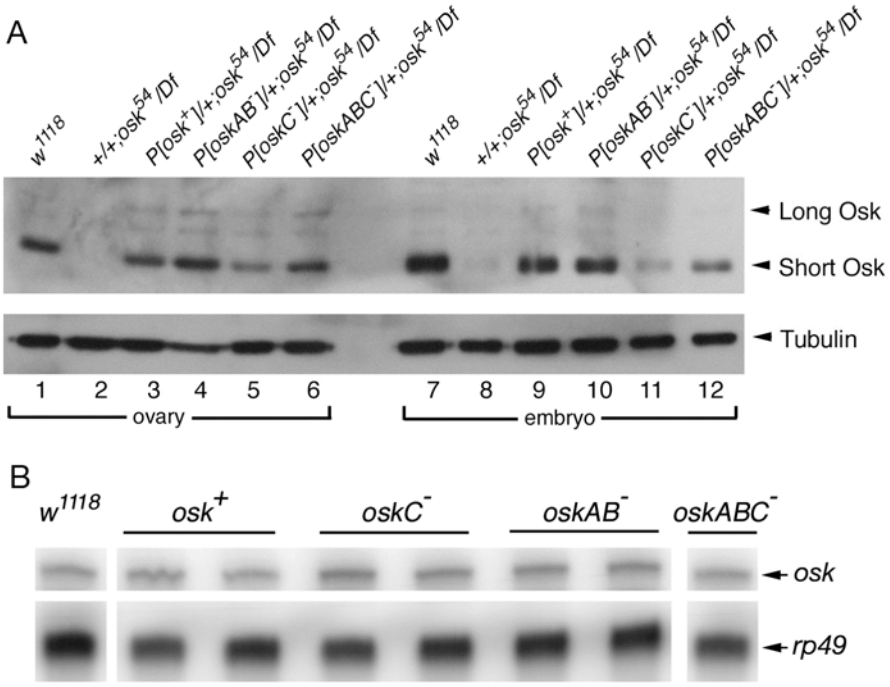

A
w1118
+/+;osk54/Df
P[osk+]/+;osk54/Df
P[oskAB-]/+;osk54/Df
P[oskC-]/+;osk54/Df
P[oskABC-]/+;osk54/Df
w1118
+/+;osk54/Df
P[osk+]/+;osk54/Df
P[oskAB-]/+;osk54/Df
P[oskC-]/+;osk54/Df
P[oskABC-]/+;osk54/Df
Long Osk
Short Osk
Tubulin
1 2 3 4 5 6
ovary
7 8 9 10 11 12
embryo
B
w1118
osk+
oskC-
oskAB-
oskABC-
osk
rp49

Figure 3.6. Assay system that selectively monitors *osk* AB region-dependent repression of translation.

Transcripts produced by the *P[UAS-osk]*, *P[UAS-osk-AB]* and *P[UAS-osk-AB⁻]* transgenes are shown in schematic form, and differ only in the presence of the ~120 nt AB region which is either wild type or bearing the BRE⁻ mutations described above. Portions of the *osk* mRNA are shown as a thick black line (5' UTR sequences in both transcripts and the *osk* AB 3' UTR region in the *UAS-osk-AB* and *UAS-osk-AB⁻* transcripts) or a shaded rectangle (coding region). The common 3' UTR (undulating line) is a part of the *fs(1)K10* mRNA which is present in the pUASp vector (Rorth, 1998) and does not include the TLS localization element (Serano and Cohen, 1995). The AB region is not sufficient to direct posterior localization (Kim-Ha et al., 1993), and as expected the *P[UAS-osk-AB]* transcripts are not localized (data not shown). The transcripts contain additional 5' UTR sequences from the pUASp vector which are not shown.

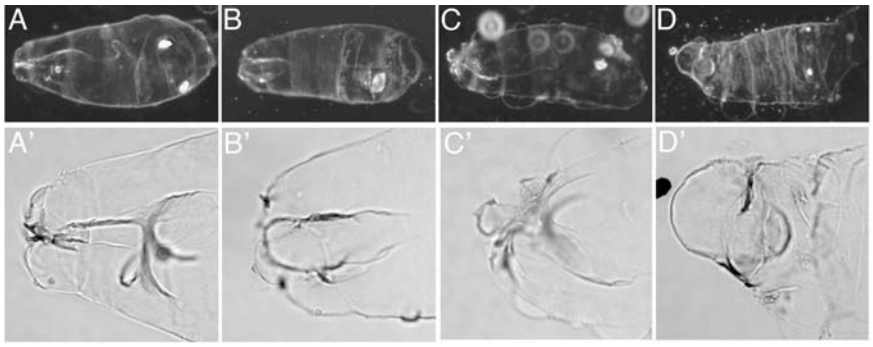
Expression of the transgenes in females with the maternal alpha tubulin GAL4 driver produces dramatically different phenotypes, as expected given the role of the AB region in translational repression. The levels of the transcripts are similar (data not shown).

Transgene	wild type (%)	anterior defects (%)	bicaudal (%)	n
P[UAS-osk]-1	7	5	88	306
-2	7	2	91	375
P[UAS-osk-AB]-1	99	1	0	239
-2	95	5	0	308
P[UAS-osk-AB⁻]-1	27	20	54	224
-2	12	27	61	243

Figure 3.7. Bru mutant protein unable to bind Cup interferes with translational repression by wild type Bru.

To selectively monitor *osk* AB-dependent repression, wild type and interaction-defective forms of Bru were expressed in *osk*$^{-}$ females also expressing the *P[UAS-osk-AB]* reporter transcript. Expression of Osk from *P[UAS-osk-AB]* interferes with anterior development, and a low level of *osk* activity owing to incomplete repression via the AB region produces ~20% of embryos with an incomplete head skeleton (weak anterior defect, panels B and C) or absence of head involution and head skeleton (strong anterior defect, panel D). Panels A-D show complete larval cuticles, while A'-D' show the head regions in greater detail. As summarized in panel E, expression of *P[UAS-bru]* in this background has little effect on patterning, while expression of *P[UAS-bru*Δ*334-416]* dramatically enhances the anterior defects. The maternal alpha tubulin GAL4 driver (abbreviated as *MAT2*) was used for expression of the UAS transgenes.

The absence of any substantial rescue of abdominal segmentation in this assay is presumably because the *UAS-osk-AB* mRNA is not localized to the posterior. Expression of an unlocalized form of *nanos* mRNA also disrupts anterior development more efficiently than it rescues posterior segmentation (Gavis and Lehmann, 1994).



E

Genotype	strong anterior defect (%)	weak anterior defect (%)	no anterior defect (%)	n
P[UAS-bruΔ334-416]/P[UAS-osk-AB] MAT2; osk^{166}/osk^{166}	28	33	39	364
P[UAS-bru]/P[UAS-osk-AB] MAT2; osk^{166}/osk^{166}	11	8	81	112
+/P[UAS-osk-AB] MAT2; osk^{166}/osk^{166}	13	10	77	119
+/+; osk^{166}/osk^{166}	0	0	100	88

Figure 3.8. BruΔ334-416 retains BRE binding activity.

(A) Binding to labeled BRE probes from the *osk* AB or C regions was measured by UV crosslinking assay with ovary extracts from wild type flies (w^{1118}) or from flies expressing *P[UAS-bruΔ334-416]* (under control of the *nosGAL4VP16* driver), as indicated. The AB and C transcripts are wild type, and the AB$^-$ and C$^-$ transcripts have point mutations in the BREs. The C RNA is larger than used previously (see Experimental Procedures), and bind better to Bru (data not shown). The larger C$^-$ RNA retains reduced but still detectable binding to Bru. BruΔ334-416 binds to both AB and C probes. Bru binds the AB probe more strongly than the C probe. This preference is lost for the BruΔ334-416 protein, a reproducible result of unknown significance.

(B) Detection of Bru proteins in ovary extracts of wild type (lane 1), *P[UAS-bru]* (lane 2) and *P[UAS-bruΔ334-416]* (lane 3)(the w^{1118} and *P[UAS-bruΔ334-416]* extracts are the same as those used in panel A). Bru and BruΔ334-416 proteins were detected by Western blotting with anti-Bru antibody. The BruΔ334-416 protein is not substantially overexpressed relative to the Bru protein from *P[UAS-bru]*.

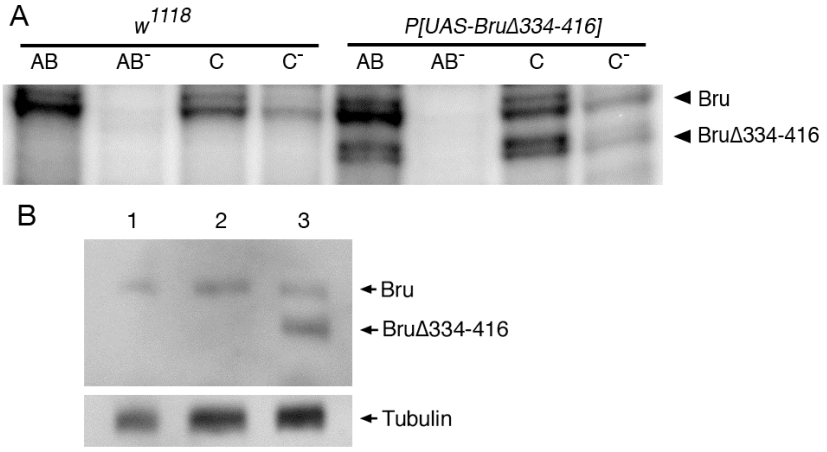
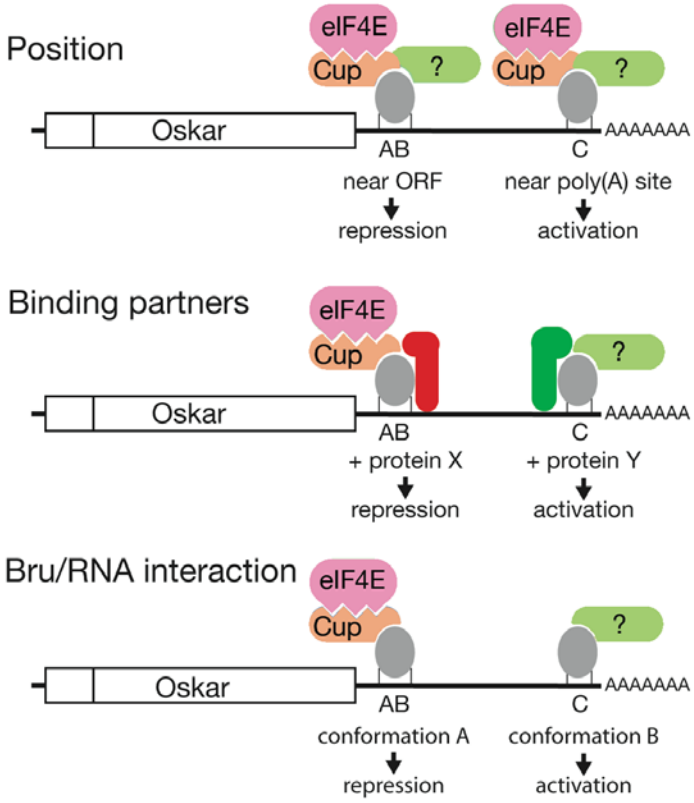


Figure 3.9. Models of Bru action as both repressor and activator of translation.

The three models are described in the text. Bru is indicated as a gray oval. A Bru-interacting protein involved in activation is indicated as ?. Postulated Bru binding partners that interact specifically with osk AB or C regions and contribute to repression and activation, respectively, are unlabeled and shown in red and green.



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Chapter 4: Bruno binds to AB and C region BREs/RNA in the *oskar* 3'UTR with distinct properties

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ABSTRACT

cis-acting elements in the 3'UTR of mRNAs often have important roles in translational regulation. Trans-acting factors can bind to these elements and regulate the expression of the mRNA. A well-established example in *Drosophila* is translational regulation of the *oskar* mRNA by Bruno. Bruno binds to two different regions (the AB and C regions) of the *oskar* mRNA 3'UTR and displays opposite functions (repression and activation) upon binding. In this study, we explored in detail how Bruno binds to its RNA targets using a quantitative assay. We find three notable features displayed by Bruno binding: high affinity, cooperative binding and apparent RNA compaction. Each of these features is evident for binding of Bru to the AB region, but binding to the C region is qualitatively and quantitatively different. These substantial differences in binding provide support for a specific model to explain the dual action of Bruno. We also find that Bru dimerization is not required for its cooperative binding to AB RNA. Instead, a complete AB region is required for all features of Bruno binding, supporting the idea that RNA substrates can influence Bruno's function.

INTRODUCTION

Many important developmental processes in Drosophila, such as body patterning, are regulated by genes that are subject to post-transcriptional regulation. This regulation often involves cis-acting elements in the 3'UTRs of the mRNAs. Some elements direct mRNA localization while others regulate translation (Johnstone and Lasko, 2001). Translational regulation can affect a variety of steps, including ribosome binding, scanning, initiation and elongation (Kuersten and Goodwin, 2003). One example is translational regulation of the *oskar (osk)* mRNA by Bruno (Bru). Bru binds to two different regions of the *osk* mRNA 3'UTR: the AB region close to the *osk* coding sequence and the C region close to the polyadenylation site (Kim-Ha et al., 1995). Data from Chapter 3 suggest that Bru has a dual role on *osk* translation: it acts as a repressor when it binds to the AB region BREs, and it acts as an activator when it binds to the C region BREs. The molecular basis for this dual action is unclear, but the nature of how Bru binds to its target sites may be an important part of the decision to repress or activate. In this study, we explore in detail the interaction of Bru with its targets.

We have proposed three different models by which Bru can have a dual role in *osk* translation. The first model is the 'binding partner model', in which additional proteins bind selectively to either the AB or C regions, and through interaction with Bru determine how Bru will function. At present, there are no candidates for such co-factors, making the model difficult to test. The second model is the 'position model', in which the relative position in the mRNA to which Bru binds determines how Bru will act. For example, proximity to the poly(A) tail might allow Bru to perform a function involving polyadenylation, while binding in a different region might preclude that function or promote another. This model is being tested by others in the lab. The third model is the

'instructive substrate model', in which the nature of the RNA substrate influences Bru's function. For example, when Bru binds to BREs in the AB region, it could adopt one conformation that recruits the repression machinery, whereas binding to BREs in the C region could lead to an alternative conformation with high affinity for the activation machinery. One fundamental difference between the last two models is that the final model posits a difference in how Bru binds the two regions, or in the effect of binding on Bru (such as its structure). All of the previous analyses of Bru/RNA binding relied on UV crosslinking assays (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995). Here we use a more quantitative method to study the binding of Bru to the AB and C regions.

We find three notable features displayed by Bru binding: high affinity, cooperative binding and apparent RNA compaction. Each of these features is evident for binding of Bru to the AB region, but binding to the C region is qualitatively and quantitatively different. In addition, we find that Bru dimerization is not required for its cooperative binding to AB RNA.

RESULTS

Quantification of Bru binding to *osk* AB RNA

We performed gel mobility shift assays to investigate the binding of Bru to RNA substrates from the *osk* 3'UTR. This assay has been successfully used by others to evaluate protein/nucleic acid binding (Batey and Williamson, 1996a; Dubey et al., 2005; Magnet and Blanchard, 2004; Melo et al., 2003; Recht and Williamson, 2004; Ryder et al., 2004). In this assay, a constant level of radiolabled AB RNA was mixed with increasing amounts of recombinant Bru protein and allowed to bind at room temperature. Bru/AB RNA complex and free AB RNA were separated on a native gel (Fig 4.1B). Quantitation of the assay determines Kd, the apparent equilibrium dissociation constant, as well as cooperativity, which is measured by the Hill coefficient. No cooperativity corresponds to a Hill coefficient of 1, while a value greater than 1 indicates positive cooperativity.

100% binding was achieved at high Bru concentrations, with Bru/AB RNA complex and free AB RNA migrating to different positions (Fig 4.1B). Only a single complex was observed with increasing amounts of Bru. The percentage of RNA bound at various Bru concentrations was determined, and the data were analyzed using the Hill equation (see Materials and Methods). Our analysis revealed that Bru has high affinity for AB RNA (Kd=39 nM). Notably, binding of Bru to AB RNA is also highly cooperative (Hill coefficient of 1.7 to 2.0).

The appearance of only a single Bru/AB RNA complex was not expected. AB RNA contains multiple Bru binding sites (Kim-Ha et al., 1995), suggesting that there would be complexes that differ in the number of molecules of Bru bound. The absence of

any intermediate binding complex raises the possibility that Bru binds to AB RNA in a rapid 'all on or all off' fashion. This type of behavior is common when binding is cooperative: the binding of the first ligand lowers the energy required for the second ligand to bind to the same substrate, and the substrate reaches full occupancy very rapidly (Edelstein, 1975). Binding of Bru to AB RNA is highly cooperative, which can explain the appearance of a single complex. An alternative explanation is that under the conditions of the assay intermediate complexes, with only a subset of the binding sites occupied, are not stably associated with the RNA during electrophoresis. Therefore only the fully occupied complex is detectable.

We also find that Bru/AB RNA complex migrates in the native gel faster than free AB RNA, a phenomenon known as band acceleration (Batey and Williamson, 1996a). Typically, if a nucleic acid has the same structure when it is both free and bound, then protein/nucleic acid complexes will migrate more slowly than free nucleic acid when subjected to native polyacrylamide gel electrophoresis (Revzin, 1989). Bru protein would be expected to retard migration of the RNA. Because we observe increased migration, it appears that a change in the structure of the RNA is responsible. Specifically, the RNA appears to be compacted upon binding of Bru. Notably, Bru does not inherently accelerate migration of all target RNAs (data below), consistent with this interpretation.

There are precedents in which compaction of an RNA upon protein binding leads to band acceleration in a gel mobility shift assay. One example involves the interaction of ribosomal protein S15 with a substrate from the central domain of 16S rRNA, which corresponds to the first step in assembly of the 30S ribosomal subunit (Batey and Williamson, 1996a; Batey and Williamson, 1996b). Binding of S15 leads to increased mobility of the RNA. Structural data show that S15 binding is coupled to a conformational change in a three-helix junction of the RNA, consistent with the

interpretation that compaction of the RNA is responsible for accelerated migration (Agalarov et al., 2000). Another example is Alfalfa mosaic virus (AMV) coat protein (CP), which is required to activate viral RNA replication in an infected cell. AMV CP shows band acceleration upon binding to a viral RNA substrate. The RNA binding domain (RBD) of the CP binds to AUGC tetranucleotide repeats in the 3'UTR of the viral RNA to form a structurally organized 3' terminus, and this change in RNA structure can account for the observed band acceleration (Guogas et al., 2004; Petrillo et al., 2005).

Bru binds differently to *osk* AB and C regions

To further investigate the reason why the binding of Bru to AB and C regions in the *osk* 3'UTR leads to opposite functions in translation, we compared these two binding events. The C RNA used was a 192 nt RNA that includes all recognizable BREs in the C region and extends to the 3' end of the *osk* 3'UTR (Fig 4.1A). Bru binds to C RNA with three times lower affinity (Kd=125 nM) compared to AB RNA (Kd=39 nM). This quantitative difference in binding of Bru to the two substrates is accompanied by qualitative differences: binding to the C RNA is not cooperative (Hill coefficient=1.1), and does not display band acceleration (Fig 4.1C).

A complete AB region is required for all features of Bru binding

The striking difference in how Bru binds AB and C RNAs raises the question of what part of the AB substrate is required for cooperative binding and the apparent compaction of the RNA. To address this question we tested mutant AB RNAs in which some but not all of the Bru binding sites were modified by point mutations or removed by terminal deletions (for a diagram see Fig 4.2A). The A and B RNAs consist of the 5' and 3' portions of AB, respectively (details see Materials and Methods). The sAB RNA

(Gunkel et al., 1998) contains only the central 80 nt of AB. The A+B- RNA includes point mutations that disrupt the Bru binding sites in the B portion of AB.

All RNAs from which some Bru binding sites are removed by deletions show low affinity for Bru. sAB RNA has Kd of 1 uM and A and B RNA have Kds of greater than 1 uM (Fig 4.2B). The Hill coefficient could not be determined due to poor binding. All three RNAs show retarded complex migration, indicating the RNAs are not compacted the same way as AB RNA is.

The RNA in which some Bru binding sites were disrupted by point mutations also displays both quantitative and qualitative differences, relative to AB RNA, in how it binds Bru. The affinity is substantially reduced, with a Kd of 256 nM (vs 39 nM for AB RNA), and binding is only weakly cooperative (Hill coefficient=1.3) (Fig 4.2B). This residual cooperativity may come from the wild type BREs in the 5' half of A+B- RNA. Binding of Bru to the A+B- RNA retards its migration, indicating that binding sites in B, or the presence of all binding sites in AB, is required for band acceleration and apparent compaction of AB RNA.

Cooperative Bru binding - Bru dimerization is not required

One of the notable features of Bru binding to AB RNA is cooperativity. Many proteins display cooperative binding for their nucleic acid substrates. Often, cooperative binding is achieved through protein homo- or hetero-dimerization. Binding of the first protein brings along or recruits the second, which can bind to the same substrate easier. Many examples of DNA binding proteins display this phenomenon (Courey, 2001; Koudelka, 2000). An RNA binding protein for which dimerization underlies cooperative binding is the poly(A)-binding protein (PABP). PABP binds to a conserved A-rich sequence present in the 5'UTR of its mRNA and represses translation. The cooperativity

of this binding is dependent on a C terminal domain of PABP that mediates dimerization (Melo et al., 2003).

Another type of cooperative binding, which is rare among studied examples, is not induced by protein/protein interaction. Instead, binding of the first protein alters the nucleic acid substrate and makes it more suitable for binding of the second protein. One example involves an interaction previously mentioned - S15 binding to an RNA from the central domain of 16S rRNA, which corresponds to the first step in assembly of the 30S ribosomal subunit. The second step in 30S subunit assembly is the binding of the S6:S18 heterodimer to the central domain of 16S rRNA. The two assembly steps display cooperativity. Biochemical and structural data show that the cooperativity can be assigned to a structural change in the RNA - this change is induced by S15 binding and facilitates binding of the S6:S18 heterodimer (Agalarov et al., 2000; Capel and Ramakrishnan, 1988; Recht and Williamson, 2004).

Bru forms dimers, and the interaction domain has been defined. To determine if dimerization is required for cooperative binding of Bru to AB RNA, we tested the RNA binding properties of a Bru mutant, BruΔ334-416, which cannot dimerize (see Chapter 3; for a diagram see Fig 4.3A). BruΔ334-416 has slightly weaker affinity for binding to AB RNA (Kd = 112 nM) compared to Bru (Kd = 39 nM). Nevertheless, the binding of BruΔ334-416 to AB RNA retains positive cooperativity, and this may actually be enhanced (Hill coefficient=3.2, Fig 4.3B). In addition, BruΔ334-416 also displays band acceleration upon binding to AB RNA as if the RNA is compacted (Fig 4.3B). Thus, dimerization is not required for cooperative binding of Bru to AB RNA. Moreover, the RNA compaction may represent the property of the AB RNA that underlies cooperativity.

DISCUSSION

We have shown that Bru binds with different properties to substrates having different biological roles. Bru binding to AB RNA is cooperative, and appears to compact the RNA, whereas Bru binding to C RNA displays neither of these two features. These substantial differences in binding provide strong support for a basic property of the 'instructive substrate' model to explain the dual action of Bru, namely, that interactions at the repressive and activating sequences are distinct and different. While our results do not prove that model, they do demonstrate that no additional factors, such as those posited in the 'binding partners' model, are required to create differences in the binding of Bru to the repressive and activating substrates. In addition, the discovery that cooperative binding to the AB RNA does not involve protein dimerization, but instead depends on the RNA and correlates with an apparent change in RNA structure, provides evidence that the RNA dictates how Bru will bind, the key feature of the 'instructive substrate' model.

Are the differences in binding of Bru to AB and C important for the decision to repress or to activate?

At present we are unable to determine if the observed differences in Bru binding to the AB and C regions dictate whether Bru will repress or activate translation. The mutations in the AB region that reduce cooperativity and disrupt the apparent compaction associated with Bru binding also substantially reduce the affinity of binding. Thus any defect in repression observed when the RNAs are tested for their effects on translation could be due either to reduced binding or to qualitatively altered binding (or both).

Although we cannot yet design an experiment that would demonstrate that cooperativity and RNA restructuring are important for repression by Bru, a simple experiment might disprove this notion. We know that addition of the AB region to an

otherwise unregulated reporter mRNA leads to its repression (see Chapter 3), and this assay could be modified to target Bru to the mRNA via a different RNA binding protein. Specifically, the reporter mRNA could be modified by addition of multiple binding sites for the bacteriophage MS2 coat protein (Nagai, 1996), and an RNA binding defective form of Bru (Harrison et al., unpublished) could be expressed as a fusion to MS2 coat protein.

Our experiments with altered forms of the AB RNA do lead to one very strong conclusion. Gunkel et al previously used the sAB mutant to argue that binding of Hrp48 to the AB region is important for repression (Gunkel et al., 1998). They presented evidence that the sAB RNA retained strong Bru binding (as measured by a UV crosslinking assay), yet was defective in repression. Because the mutant RNA had substantially reduced Hrp48 binding, they concluded that binding of Hrp48 is important for repression. Our quantitative binding data clearly show that the sAB RNA has dramatically reduced affinity for Bru (Kd of 1 uM), and so the observed defects in repression are expected simply from loss of Bru binding. Although there is genetic evidence that reduction of Hrp48 activity affects *osk* mRNA translation (Yano et al., 2004), this may or may not involve binding of Hrp48 to the *osk* mRNA.

Specificity of Bru binding sites

Bru appears to be relatively selective in its action. There are three known or suggested substrates: *osk* mRNA, *grk* mRNA and *cycA* mRNA (Filardo and Ephrussi, 2003; Kim-Ha et al., 1995; Sugimura and Lilly, 2006; Webster et al., 1997). Although this may not represent all target mRNAs, Bru does not bind to many control RNAs used in binding assays.

Bru binding sites appear to have relatively low complexity. A BRE as originally defined contains 7 nt, U(G/A)U(A/G)U(G/A)U (Kim-Ha et al., 1995). Bru is very closely

related to EDEN-BP and Bru-3, with ~60% identity in amino acid sequence within the RRM RNA binding motifs (Delaunay et al., 2004; Paillard et al., 1998; Webster et al., 1997). This suggests that the proteins may have very similar binding specificities. Indeed, EDEN-BP and Bru-3 bind to repeated copies of the tetranucletides UGUA and/or UAUG (Delaunay et al., 2004; Paillard et al., 1998). These tetranucleotides appear within the BRE sequence, and are highly enriched in the *osk* AB and C regions, and may thus comprise the basic Bru binding site. If the Bru binding site is indeed such tetranucleotides, then binding sites for Bru are expected to appear in random sequences every 256 nt (or 128 nt if both tetranucleotide sequences bind Bru), and should be present in effectively all mRNAs. How then would Bru display specific binding, and how would its action be limited to only a subset of mRNAs? Part of the answer seems clear: strong binding probably requires a local concentration of the tetranucleotides. The unusual behavior of Bru in binding to the AB region raises the possibility that repression may require that multiple copies of the basic binding sites be presented in a particular organization, perhaps to promote cooperativity and/or restructuring upon binding. If so, then an 'active' arrangement of Bru binding sites might have an extremely low probability of occurring in random sequences. Further characterization of the binding of Bru to the other substrates, from the *grk* and *cycA* 3' UTRs (Filardo and Ephrussi, 2003; Sugimura and Lilly, 2006; Webster et al., 1997), should help clarify this issue.

MATERIALS AND METHODS

RNA preparation and purification

All radiolabled RNAs were prepared by in vitro transcription of cloned templates containing appropriate fragment of the *osk* 3'UTR, and subsequently purified on a denaturing RNA acrylamide gel followed by a protocol from Ambion (Technical Bulletin #171). The template for both AB and A probes was generated previously by subcloning an EcoRI-DraI fragment from *osk* 3'UTR into pSP72 vector (Kim-Ha et al., 1995) (DNA template was linearized with BglII and ApaLI for transcription of AB and A RNA, respectively). The B template was generated by subcloning an ApaLI-DraI fragment into pSP73 (DNA template was linearized with DraI for transcription of B RNA). The A+B- template was generated by replacing the ApaLI-DraI fragment of AB(BRE+) with the equivalent fragment from AB(BRE-) (Kim-Ha et al., 1995) (DNA template was linearized with BglII for transcription of A+B- RNA). The sAB template (Gunkel et al., 1998) was generated by PCR (primers: 5'-cgggatccgtagtatgttctctgtctttg-3' and 5'-gaagatctcaataatatacaataatggactag-3') and subcloned into BamHI and BglII sites of pGEM2 (DNA template was linearized with SacI for transcription of sAB RNA). The C template was generated by PCR (primers 5'-tctggatccttctggcgtaatttacagc-3' and 5'-tcggatcccagttactttgaacatagc-3') and subcloned into pSP72 as an XbaI (a native site on the *osk* 3'UTR) and BamHI fragment (DNA template was linearized with BamHI for transcription of C RNA).

Protein constructs and purification

For expression of 6xHisBru and 6xHisBruΔ334-416, wild type Bru coding regions was generated by joining two fragments, NdeI (start codon)-EcoRV fragment by

PCR (primers: 5'-ccgaattcatatgttcaccagccgcgctt-3' and 5'-ccgaattctagcgttcattgcgattctcgc-3') and EcoRV-EcoRI (stop codon) fragment from a *bru* cDNA clone (Webster et al., 1997). It was then subcloned into pET15b (Promega) as an NdeI-EcoRI fragment for 6xHis fusion at the N terminus. The BruΔ334-416 coding region was generated from by deleting 83 amino acids from the full length Bru coding region using two unique restriction sites, PflMI and NotI, followed by blunt end ligation. 6xHisBru and 6xHisBruΔ334-416 were expressed in Codonplus RP and pLysS *E. coli* (Stratagen), respectively. The bacteria were grown to ~0.7 optical density (measured at 600 nm), and induced by addition of IPTG to 1 mM. 6xHisBru was induced at room temperature for 6 hrs because of its insolubility. 6xHisBruΔ334-416 was induced at 37°C for 3 hrs. Bacteria cultures were collected as pellets in 50 mL Falcon tubes by centrifugation at 5000g in the cold. Pellets were frozen at –20°C and thawed on ice on the next day. Pellet from 50 mL of culture was resuspended in 5 mL Histag Binding Buffer (20 mM phosphate buffer pH 7.8, 500 mM NaCl, 10% glycerol, 1xProtease Inhibitors (500 uM pefabloc (Boehringer Mannhelm), 2.5 mM benzamidine, 10 ug/mL pepstatin, and 10 ug/mL leupeptin), 20 mM Imidazole). Resuspended culture was sonicated on ice and cell debris was pelleted in a glass tube at 4000g for 15 min at 4°C. The supernatant was saved as lysate and kept on ice until loading onto a purification column. A purification column was made by packing 1.5 mL Prebond Histag Resin (Invitrogen) in a 3 mL clinical syringe. It was then assembled onto a Biorad ECONO Gradient Pump in the cold room. The column was pre-washed with dH_2O for 20 mins and then Histag Binding Buffer for another 20 mins (1 mL/min, same below). Lysate was loaded onto the column and the following gradient program was used for purification: 10min/0%B, 20min/0-100%B, 10min/100%B (A-20 mM Imidazole in Histag binding buffer; B-300 mM Imidazole in

Histag binding buffer). About 40 1 mL fractions were collected using a Biorad Fraction Collector (Model 2110).

Aliquots of each fraction (20 μl) were run on a SDS-PAGE followed by coomassie staining to determine the peak fractions. Peak fractions were pooled and concentrated by dialysis against 25% PEG (MW 15-20,000 PEG in 25 mM Tris pH 7.5, 1 mM EDTA, 200 mM KCl) followed by dialysis against 1x GMS Binding Buffer (10 mM Tris-Cl pH 8.0, 25 mM NaCl, 0.1 mM EDTA, 1x protease inhibitors, 10% glycerol) overnight. Protein concentrations were determined by UV spectroscopy using NanoDrop (NanoDrop Technologies). Extinction coefficients for 6xHisBru and 6xHisBruDA were estimated by the method of Gill and von Hippel (available at http://us.expasy.org/tools/protparam.html). Proteins were then diluted with 1x GMS Binding Buffer to 10 uM for use as a 10x stock.

Electrophoretic gel mobility shift assays and data analysis

The complex between protein and RNA was visualized by gel mobility shift assay. The assay was carried out as described (Ryder et al., 2004) with some modifications. The final equilibration reactions contain 1x GMS Binding Buffer, 0.1 mg/mL tRNA, 5 ug/mL heparin, 0.5% NP-40, 50 ug/mL BSA, 200 pM radiolabeled RNA and protein concentrations ranging from 0 to 1000 nM. The RNA was heated to 55°C for 5 mins and cooled at room temperature for 5 mins. Total reaction volumes were 20 uL. Reactions were kept at room temperature for 2 hours. During the same period of time, a native polyacrylamide gel (6% w/v 37.1:1 acrylamide/Bis-acrylamide, 0.25xTBE, 0.75 mm thick) was pre-run at 400V in the cold room also for 2 hours. Before loading, 4 ul of loading dye (30% v/v glycerol, 0.05% w/v xylene cyanol) was added to each sample. A portion (10 uL) of each reaction was loaded onto the pre-run gel and continued to run at

400V for another 3 hrs in the cold room. Gels were dried and exposed to a PhosphorImager screen overnight (Molecular Dynamics).

Data were quantified using ImageQuant software (Molecular Dynamics) and the fraction of bound RNA was determined as Bound%=Bound/(Free+Bound) for each protein concentration. Bound% was plotted against protein concentration using KaleidaGraph (v4.0). Apparent dissociation constants (Kd) for titration of RNA with protein were determined using the equation y=m0^m2/(m0^m2+m1^m2), where y is the fraction of bound RNA, m0 is protein concentration, m1 is the Kd and m2 is the Hill coefficient.

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FIGURES

Figure 4.1. Bru binds differently to *osk* AB and C regions.

A. A diagram of the *osk* mRNA. The *osk* coding sequence is shown as a thick dark bar. AB and C regions (also RNAs) are shown as thin dark bars right above a thin dark line representing the *osk* 3'UTR. Diagram is not drawn to scale. B. Binding of Bru to radiolabeled AB RNA monitored by gel mobility shift assay. Migrating positions of free AB RNA and the Bru/AB RNA complex are labeled as Free and Bound on the right, respectively (same below). Bru protein concentrations range from 0-1000 nM (1:1 dilution series, see Materials and Methods). Each lane corresponds to the following Bru concentrations (from left to right, same to all gel panels): 0, 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000 nM. Plot of the fraction of AB RNA bound versus the concentration of Bru are also shown. Data were fit using non-linear least squares analysis as described in Materials and Methods. Kd is 39 nM and Hill Coefficient is 1.7, indicating positive cooperativity. Note that Bru accelerates AB RNA to a faster migrating position comparing to free AB RNA. C. Binding of Bru to radiolabeled C RNA monitored by similar gel mobility shift assay as in B. The data were also analyzed the same way as described in B. Kd is 126 nM and Hill Coefficient is 1.1, indicating non-cooperative binding. In contrast to AB RNA, binding of Bru retards the migration of C RNA. Extents of AB (126 nt) and C RNA (192 nt) are described in Materials and Methods.

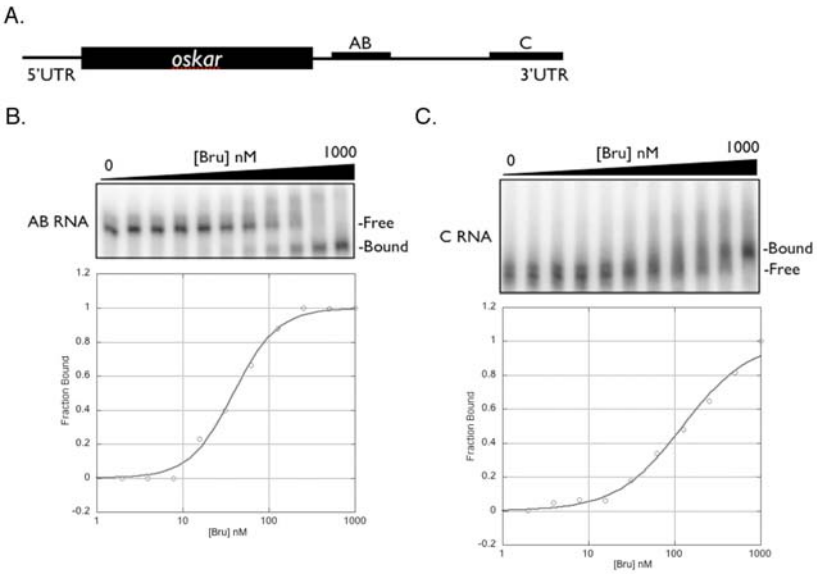

A.
AB
C
oskar
5'UTR
3'UTR
B.
0
[Bru] nM
1000
AB RNA
-Free
-Bound
Fraction Bound
[Bru] nM
C.
0
[Bru] nM
1000
C RNA
-Bound
-Free
Fraction Bound
[Bru] nM

Figure 4.2. A complete AB region is required for all features of Bru binding.

A. A diagram of wild type AB RNA and four modified RNAs: sAB, A, B and A+B-. Dark box indicates a BRE. Diagram is not drawn to scale. Details about these five RNAs are described in Results or Materials and Methods. B. Binding of Bru to radiolabled RNAs in gel mobility shift assays. RNAs used in the assay are indicated on the left. Kds for each Bru/RNA interactions are shown on the right. Bru concentration in each lane is the same as in Fig 4.1.

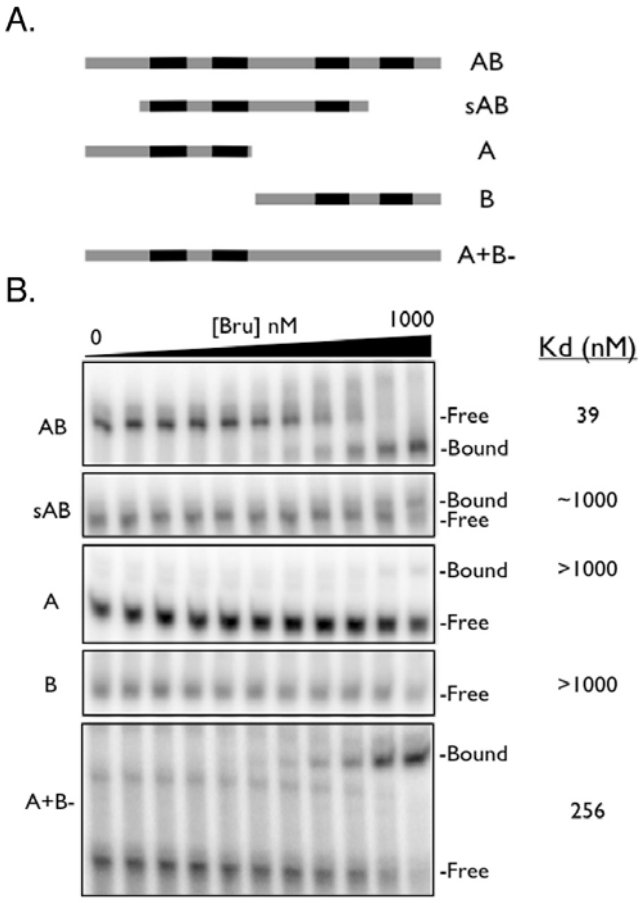
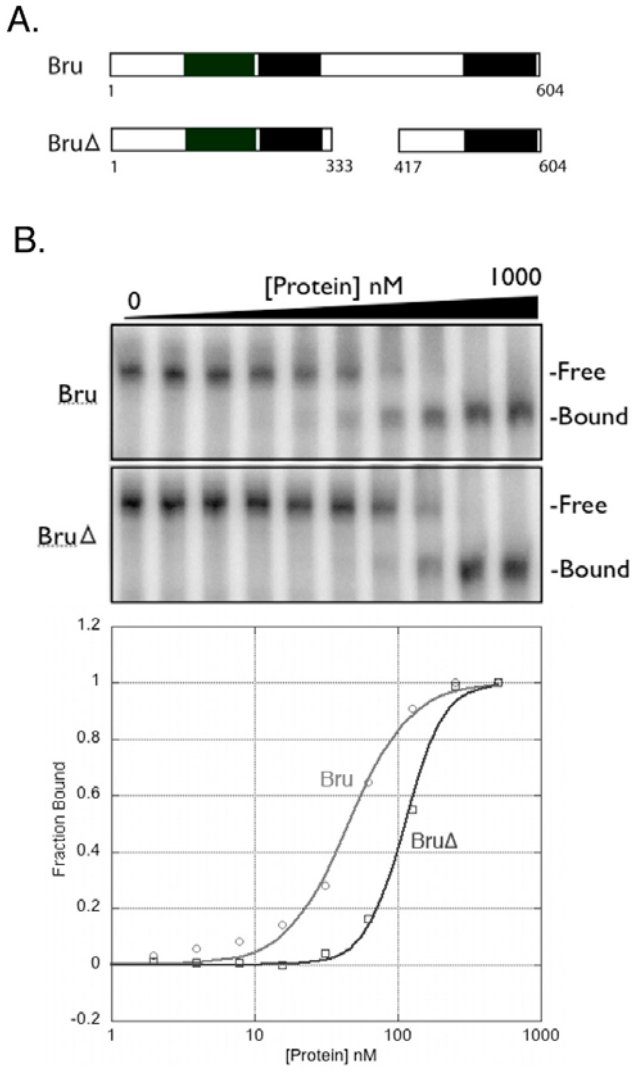


Figure 4.3. Bruno dimerization is not required for cooperative binding to the AB RNA. A. A diagram of wild type Bru and dimerization-defective mutant BruΔ334-416 (BruΔ in the figure and below). Dark box indicates a RRM. B. Binding of Bru or BruΔ to radiolabeled AB RNA monitored by gel mobility shift assay. Both panels in B are from the same native gel. Data were plotted in B. Bru/AB RNA binding – Kd is 45.7 nM and Hill Coefficient is 2.0. BruΔ/AB RNA binding – Kd is 112.5 nM and Hill Coefficient is 3.2. Note that BruΔ can still bind to AB RNA cooperatively. Each lane corresponds to the following Bru concentrations (from left to right): 0, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000 nM.



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